



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 15/12, 15/11, 5/10, A01K 67/027, C07K 14/705, A61K 48/00, 49/00, G01N 33/50, C12Q 1/68, A61K 39/395, A61P 3/06</b>		A2	(11) International Publication Number: <b>WO 00/47735</b>																																																		
			(43) International Publication Date: <b>17 August 2000 (17.08.00)</b>																																																		
<p>(21) International Application Number: <b>PCT/CA00/00145</b></p> <p>(22) International Filing Date: <b>8 February 2000 (08.02.00)</b></p> <p>(30) Priority Data: 60/119,024 8 February 1999 (08.02.99) US</p> <p>(71) Applicant (for all designated States except US): <b>MCGILL UNIVERSITY [CA/CA]; 3550 University Street, Montreal, Quebec H3A 2A7 (CA).</b></p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): <b>GIGUERE, Vincent [CA/CA]; 277 Querbes, Outremont, Quebec H2V 3W1 (CA). SLADEK, Robert [CA/CA]; 3659 Ste-Famille, Montreal, Quebec H2X 2L5 (CA). LUO, Jiang-Ming [CA/US]; 1626 North Fig Avenue, Marshfield, WI 54449 (US).</b></p> <p>(74) Agents: <b>DUBUC, Jean, H. et al.; Goudreau Gage Dubuc, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).</b></p>																																																					
<p>(81) Designated States: <b>AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>																																																					
<p>(54) Title: <b>NON-HUMAN TRANSGENIC ANIMAL WHOSE GERM CELLS AND SOMATIC CELLS CONTAIN A KNOCKOUT MUTATION IN DNA ENCODING ORPHAN NUCLEAR RECEPTOR ERR<math>\alpha</math>lpha</b></p>																																																					
<table border="1"> <caption>Estimated data for Figure 1: Body weight (g) vs Weeks after birth</caption> <thead> <tr> <th>Weeks after birth</th> <th>WT Female (g)</th> <th>KO Female (g)</th> <th>WT Male (g)</th> <th>KO Male (g)</th> </tr> </thead> <tbody> <tr><td>0</td><td>5</td><td>5</td><td>5</td><td>5</td></tr> <tr><td>2</td><td>8</td><td>7</td><td>8</td><td>7</td></tr> <tr><td>4</td><td>12</td><td>11</td><td>12</td><td>11</td></tr> <tr><td>6</td><td>16</td><td>15</td><td>16</td><td>15</td></tr> <tr><td>8</td><td>19</td><td>18</td><td>19</td><td>18</td></tr> <tr><td>10</td><td>22</td><td>21</td><td>22</td><td>21</td></tr> <tr><td>12</td><td>24</td><td>23</td><td>24</td><td>23</td></tr> <tr><td>14</td><td>26</td><td>25</td><td>26</td><td>25</td></tr> <tr><td>16</td><td>28</td><td>27</td><td>28</td><td>27</td></tr> </tbody> </table>				Weeks after birth	WT Female (g)	KO Female (g)	WT Male (g)	KO Male (g)	0	5	5	5	5	2	8	7	8	7	4	12	11	12	11	6	16	15	16	15	8	19	18	19	18	10	22	21	22	21	12	24	23	24	23	14	26	25	26	25	16	28	27	28	27
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<p>(57) Abstract</p> <p>The present invention relates to a transgenic non-human mammal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR<math>\alpha</math>. More specifically, the invention relates to knockout mice whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR<math>\alpha</math>. The invention further relates to such knockout non-human mammals which express an ERR<math>\alpha</math> gene which is different from the endogenous gene which was disrupted. In a particular embodiment, the invention relates to a transgenic mouse having its endogenous ERR<math>\alpha</math> gene disrupted and expressing human ERR<math>\alpha</math>. As well, the invention relates to cell lines in which ERR<math>\alpha</math> activity (and/or level) has been inactivated or augmented. The invention further relates to uses and methods of the transgenic animals of the present invention to select agents which modulate the expression and/or activity of ERR<math>\alpha</math>.</p>																																																					

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**TITLE OF THE INVENTION**

NON-HUMAN TRANSGENIC ANIMAL WHOSE GERM CELLS AND SOMATIC CELLS CONTAIN A KNOCKOUT MUTATION IN DNA ENCODING ORPHAN NUCLEAR RECEPTOR ERR $\alpha$

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**FIELD OF THE INVENTION**

The present invention relates to a transgenic non-human animal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR $\alpha$ . More particularly, the 10 invention relates to a non-human transgenic mammal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR $\alpha$  and more specifically to a transgenic mice whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR $\alpha$ . In one particular embodiment, 15 mice containing a disruption of both copies of the ERR $\alpha$  gene lack detectable expression of the ERR $\alpha$  protein. The invention further relates to such knockout non-human animals which express an Err $\alpha$  gene which is different from the endogenous gene which was disrupted. In a particular embodiment, the invention relates to a transgenic mouse 20 having its endogenous ERR $\alpha$  gene disrupted and expressing human ERR $\alpha$ . As well, the invention relates to cell lines in which ERR $\alpha$  activity (and/or level) has been inactivated or augmented. The invention further relates to uses and methods of the transgenic animals of the present invention to select agents which modulate the expression and/or activity 25 of ERR $\alpha$  and to agents identified by these methods.

## BACKGROUND OF THE INVENTION

The orphan nuclear receptor estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) was initially cloned by low stringency screening of human kidney library using the estrogen receptor (ER $\alpha$ ) DNA-binding domain as a probe (Giguère et al., 1988). Although ERR $\alpha$  displays significant homology to ER $\alpha$ , it does not bind estrogens *in vitro*, nor is its transcriptional activity modulated by estrogens (Giguère et al., 1988; Yang et al., 1996). ERR $\alpha$  binds to hormone response elements containing a single consensus half site flanked by the 5' upstream sequence TNA as well as to consensus estrogen response elements (Bonnelye et al., 1997; Johnston et al., 1997; Sladek et al., 1997). Recent studies performed *in vitro* have implicated ERR $\alpha$  in a wide variety of physiologic processes, including adipocyte development (Sladek et al., 1997), cellular fatty acid oxidation (Sladek et al., 1997; Vega and Kelly, 1997), bone development (Bonnelye et al., 1997), steroidogenesis (Yang et al., 1998) as well as in thyroid hormone receptor isoform expression (Vanacker et al., 1998). In addition, ERR $\alpha$  has been shown to heterodimerize with ER $\alpha$  in solution and can modulate the estrogen responsiveness of the lactoferrin gene promoter (Yang et al., 1996).

Obesity is a prevalent disorder that often leads to diabetes, cardiovascular disease, and joint disorders. Although the precise mechanism which leads to the development of obesity has yet to be precisely determined, it appears clear that a number of mechanisms, which normally function to maintain homeostasy and normal body weight are involved. Transgenic mice with an induced brown fat deficiency have indicated that this tissue is implicated in the control of the balance of in mice (Lowell et al., Nature 366:740-742, 1993). Further, a correlation between brown adipose tissue dysfunction and obesity and diabetes has

been reported (Lowell et al., *Supra*). Previous studies have demonstrated that ERR $\alpha$  is highly expressed in brown adipose tissue (BAT) during murine development and that the receptor is upregulated during white and brown adipocyte differentiation *in vitro* (Sladek et al., 1997; Vega and Kelly, 1997). In addition, ERR $\alpha$  has been shown to modulate the activity of the medium chain acyl-coA dehydrogenase (MCAD) promoter, a key regulatory step in the fatty acid  $\beta$ -oxidation pathway (Sladek et al., 1997; Vega and Kelly, 1997). More recently, a transgenic mouse whose germ cells and somatic cells contain a knockout mutation in DNA encoding an endogenous  $\beta$ -adrenergic receptor polypeptide, thereby obtaining a mouse having a modest increase in body fat, has been reported (US 10 5,789,654).

There thus remains a need to identify the physiological function of ERR $\alpha$  *in vivo*. There also remains a need to better identify 15 which homeostatic mechanism, when disrupted or malfunctioning is implicated in the development of obesity and related diseases. In addition, there remains a need to provide animal models of obesity and related diseases, and model systems which can enable the identification and selection of agents which modulate the pathways implicated in the 20 development of obesity and related diseases. Furthermore, there remains a need to identify a target for the eventual therapy of obesity and related diseases.

The present invention seeks to meet these and other needs. Indeed, in order to identify the precise physiological function of 25 ERR $\alpha$  *in vivo*, a new strain of mice is herein provided, in which ERR $\alpha$  function has been ablated by homologous recombination in embryonic stem cells. The present invention, in particular, relates to this new strain of mice and to the function of ERR $\alpha$  and related factors *in vivo*.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

5 **SUMMARY OF THE INVENTION**

In general, the present invention relates to ERR $\alpha$ -deficient non-human transgenic mammals. More specifically, the invention relates to a transgenic non-human mammal whose germ cells and somatic cells contain a knockout mutation in DNA encoding the 10 ERR $\alpha$  endogenous orphan nuclear receptor polypeptide. In one embodiment, the transgenic mammal also includes germ cells and somatic cells expressing DNA encoding a non-endogenous ERR $\alpha$  orphan nuclear receptor polypeptide. In a preferred embodiment, the transgenic mammal also includes germ cells and somatic cells expressing DNA 15 encoding human ERR $\alpha$  orphan nuclear receptor polypeptide.

Also in general, the present invention relates to the surprising demonstration that ERR $\alpha$  is implicated in lipogenesis (fatty acid synthesis), fatty acid esterification (triglyceride synthesis), and fatty acid oxydation. Indeed, the ERR $\alpha$  knockout mouse of the present invention 20 displays abnormalities in lipogenesis, fatty acid esterification and fatty acid oxydation. The present invention therefore provides the means to affect these three processes. The knockout mammal of the present invention also demonstrates that the alteration of the activity of ERR $\alpha$  affects weight gain in an animal.

25 In a further general aspect, the invention relates to ERR $\alpha$  as a target to regulate lipogenesis, fatty acid esterification and fatty acid oxydation *in vivo*. ERR $\alpha$ , cell lines and animals of the present invention can now be used to screen for regulators of ERR $\alpha$  activity and

level. The present invention thus provides the means to identify small diffusible ligands which can modulate the activity of the putative steroid hormone receptor ERR $\alpha$ .

Based on the results presented herein, the inhibition of 5 ERR $\alpha$  activity is relevant to the treatment of glucose metabolism disorders as well as obesity.

Until the present invention, studies of ERR $\alpha$  and its role in cellular physiology were limited to *in vitro* studies and studies in culture cells, or extracts thereof. Therefore, such studies did not assess the 10 action of ERR $\alpha$  and interacting factors on metabolic pathways dependent on such interactions, which could result in a physiologically significant effect such as, for example, lipogenesis, fatty acid esterification, fatty acid oxydation, and metabolic process controlling energy balance and adiposity in a living animal or preferably in a living mammal.

15 Prior to the present invention, there had been no demonstration or suggestion that ERR $\alpha$  could have such a significant lipid metabolism, or weight gain, and/or glucose metabolism. In view of the complexity of such physiological pathways, and of the complexity of the transcription machinery operating at estrogen receptor cis-acting 20 sequences (for example, see Sladek et al., 1997) and the fact that ERR $\alpha$  interacts with ERR $\beta$  and/or with ERR $\gamma$ , to modulate transcription promoters comprising such cis-acting elements, there was no teachings or suggestions that a knockout of ERR $\alpha$  could have such a significant impact on the metabolism of an animal. Indeed, in view of the complexity 25 of the interaction of the interacting factors binding to estrogen receptor cis-elements and related cis-elements, to modulate promoter activity of different genes, it could not be reasonably predicted that a knockout of

ERR $\alpha$  would not be compensated by other factors which interact therewith (e.g. ERR $\beta$  or ERR $\gamma$ ).

In addition, the invention relates to a method of producing a transgenic non-human mammal displaying a lean phenotype  
5 the non-human mammal lacking expression of the endogenous ERR $\alpha$  orphan nuclear receptor polypeptide, the method including a disruption of the DNA encoding ERR $\alpha$ , and a selection of progeny whose germ cells and somatic cells contain a knockout mutation in DNA encoding ERR $\alpha$ , thereby yielding a lean non-human transgenic animal. Of course, such  
10 lean transgenic animals could also be produced using a reduced amount of ERR $\alpha$  (e.g. using antisense ERR $\alpha$ , for example), as opposed to a total abrogation of its expression. In addition, animals expressing nucleic acid sequence which enables an inhibition of the interaction between ERR $\alpha$  and interacting factors (e.g. cis-response elements and the like) could  
15 also be produced.

In a preferred embodiment, the invention relates to transgenic mice homozygous for the ERR $\alpha$  mutation, the mice being viable and fertile but exhibiting lipoatrophy despite normal food intake, fat absorption and metabolic activities. The ERR $\alpha$ -deficient lean mice have  
20 higher levels of circulating free fatty acids, and the mutant liver, gut and adipose tissue displayed reduced lipogenesis, fatty acid esterification, and fatty acid oxydation, contributing to the lean phenotype.

Furthermore, the present invention relates to the demonstration that ERR $\alpha$  is required for the regulation of lipogenesis, fatty acid esterification and oxydation and metabolic processes controlling energy balance and adiposity, thereby providing a new target for the development of therapeutics for obesity, fat deposition disorders and related diseases, such as diabetes. The present invention further relates  
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to ERR $\alpha$  as a target for the development of diagnostics for obesity, fat deposition disorders and related diseases.

The present invention further relates to ERR $\alpha$ -deficient non-human mammals as a new model for the investigation of lipid metabolism and associated diseases.

The ERR $\alpha$ -deficient mice of the present invention demonstrate that ERR $\alpha$  is required for the regulation of lipogenesis and metabolic processes controlling energy balance and adiposity and suggest that pharmacologic modulation of ERR $\alpha$  activity may provide means to control obesity in humans. The present invention therefore provides a new model for the investigation of lipid metabolism and associated diseases.

In another aspect, the invention features a method of producing a transgenic non-human mammal capable of expressing a functionally active non endogenous ERR $\alpha$  polypeptide, the non-human mammal lacking expression of the endogenous ERR $\alpha$  polypeptide, the method including: (a) providing a transgenic non-human mammal whose germ cells and somatic cells are deficient in ERR $\alpha$  (i.e. ERR $\alpha$  knockout); (b) introducing a non-endogenous ERR $\alpha$  transgene capable of expressing a ERR $\alpha$  polypeptide, into a cell of the non-human mammal; and (c) obtaining progeny expressing the non-endogenous transgene. In a preferred embodiment, the non endogenous ERR $\alpha$  transgene is a human transgene. In an especially preferred embodiment, the non endogenous transgene will be expressed in obesity-implicated cells and tissues.

Thus, the present invention relates to a knock-in approach, by which a wild type or mutant copy of the ERR $\alpha$  gene (i.e. human) is introduced or replaces the disrupted copy of the endogenous

ERR $\alpha$  gene. The knock-in approach has been described (Hanks et al. (1995) *Science* 269:679-682) and has been shown to enable the expression of the non-endogenous copy of the gene in the same cells as that of the endogenous gene.

5                   In a related aspect, the present invention relates to the use of such non-human transgenic mammals expressing a non-endogenous ERR $\alpha$  transgene to screen for a compound or agent that modulates ERR $\alpha$  orphan nuclear receptor activity, the method including: exposing the non-human transgenic mammal of the invention to the  
10 candidate compound, and determining the activity of the ERR $\alpha$  orphan nuclear receptor in the mammal, wherein an increase in the receptor activity as compared to untreated non-human mammals is indicative of a compound being capable of increasing ERR $\alpha$  orphan nuclear receptor activity, while a decrease in the receptor activity as compared to  
15 untreated non-human mammals is indicative of a compound being capable of decreasing ERR $\alpha$  orphan nuclear receptor activity. In a preferred embodiment, the method further includes a determination of body or physiology parameters. Non-limiting examples thereof comprise a determination of: mass, body temperature, body fat content, fat to lean  
20 mass ratio, white adipose tissue deposits, basal metabolic rate, food intake, hepatic synthetic functions, fasting serum triglyceride, serum glucose levels, level of expression of uncoupling protein mRNA in brown adipose tissue (BAT) and skeletal muscle, adipocyte volume in fat pads, lipogenesis, and fatty acid esterification and oxydation.

25                   As it will be understood by the person of ordinary skill, the present invention provides a number of significant advantages. For example, as for transgenic animals in general which have been shown to be useful for the investigation of biological processes and as animal

model systems for general and specific aspects of health sciences in humans, the transgenic animals of the present invention provide a significant and pertinent model system for screening drugs to isolate therapeutic agents. In a particular embodiment, the novel transgenic 5 animals of the present invention enable the selection and identification of modulators of the expression and/or activity of the ERR $\alpha$  orphan nuclear receptor. In a preferred embodiment, these agents have a use as anti-obesity, anti-fat deposition disorders, and/or anti-metabolic diseases associated with fat deposition disorders agents.

10 It will also be apparent to the person of ordinary skill, to which this application pertains, that the transgenic animals of the present invention can further be bred with other animals harboring known genotypes associated with fat deposition phenotypes and related disorders. Similarly the transgenic mammals of the present invention can 15 be used in biochemical experiments and the like designed to further understand, dissect and/or treat obesity and related disorders.

15 It will also be apparent that the cells and tissues of the transgenic animals of the present invention can be useful in *in vitro* methods relating to fat deposition and related disorders (including rational 20 design and/or screening of compounds which can modulate expression and/or activity of the ERR $\alpha$  orphan nuclear receptor. In a related aspect, the present invention further relates to cell lines in which the activity of ERR $\alpha$  has been inactivated or augmented. In addition to being derived 25 from the transgenic animals of the present invention, such cell lines, can for example be derived as commonly known in the art using the construct of the present invention or derivatives or variants thereof. Such cell lines can be used similarly to the animals of the present invention to identify compounds which modulate ERR $\alpha$  level and/or activity, dissect the

physiological and biochemical function (including structure/function relationships, as they relate to fat deposition and the like) of ERR $\alpha$ . Thus, the present invention also relates to established cell lines or primary cells derived from an animal of the present invention. In one 5 embodiment, fat pads from a transgenic mouse of the present invention was used to obtain primary cells which were grown and used in *in vitro* methods (i.e. insulin effect, glucose uptake, lipogenesis measurements and the like). Such experiments validated these cells as a pertinent tool for the methods and uses of the present invention.

10 Having determined that ERR $\alpha$  is involved in fat deposition and related disorders, as described herein, the present invention identifies ERR $\alpha$  as a target for therapy and diagnosis of fat deposition and related disorders. Further, the present invention provides the means to modulate the activity of ERR $\alpha$ . For example, antisense to 15 ERR $\alpha$  can be used to decrease or abrogate the expression of ERR $\alpha$  polypeptide. This is expected to be associated with a lean phenotype. Antibodies, peptides, steroid-like compounds, pharmaceutical ligands, antagonists of ERR $\alpha$  receptor, and the like could be used with the same effect on the modulation of receptor ERR $\alpha$  activity. Alternatively, in 20 certain embodiments, the fat deposition could be increased by for example overexpressing ERR $\alpha$  in cells or tissues. Of course, the non-limiting agents mentioned above could also act as stimulators or agonists of ERR $\alpha$  receptor activity.

25 Although the instant description focuses on mammalian transgenic animals, the present invention may also find utility in less common transgenic animals such as transgenic poultry. The production of leaner poultry might also be an advantage in the meat industry.

Having now identified ERR $\alpha$  as a target for fat tissue growth modulation, glucose metabolism, fat modulation, weight gain and the like, the present invention opens the way to the identification of further targets in the same pathway. Non-limiting examples of such targets 5 include ERR $\beta$ , ERR $\gamma$ , genes encoding enzymes involved in lipid metabolism whose expression is modulated by ERR $\alpha$  and related family members.

In accordance with the present invention, there is thus provided a non-human transgenic animal whose germ cells and somatic 10 cells contain a knockout mutation in the endogenous ERR $\alpha$  orphan nuclear receptor gene, and wherein the transgenic animal shows a phenotype of an altered fat and/or glucose metabolism as compared to a control animal.

In accordance with the present invention, there is also 15 provided a method of producing a non-human transgenic animal, in which at least some cells thereof contain an altered gene encoding an altered ERR $\alpha$ . The altered gene has been targeted to disrupt the endogenous ERR $\alpha$  gene in the transgenic animal. The method comprises:

- 20 a) providing an altered gene encoding the altered form of ERR $\alpha$  and designed to target and disrupt the endogenous ERR $\alpha$  gene of an embryonic stem cells (ES) of the animal;
- b) introducing the altered gene in the ES cells;
- c) selecting ES cells in which the altered ERR $\alpha$  gene has disrupted the endogenous ERR $\alpha$  gene;
- d) injecting the selected ES cells of c) into blastocysts;
- e) implanting the blastocysts of d) in a pseudopregnant animal; and

f) producing a non-human transgenic animal having at least some cells having the altered  $\text{ERR}\alpha$  gene encoding the altered  $\text{ERR}\alpha$ .

In addition, in accordance with the present invention, 5 there is also provided a method of producing the non-human transgenic animal of the present invention. The method comprises:

(a) providing a non-human transgenic animal lacking detectable levels of  $\text{ERR}\alpha$  orphan nuclear receptor gene and exhibiting a lean phenotype;

10 (b) introducing a non endogenous  $\text{ERR}\alpha$  orphan nuclear receptor transgene encoding a functional  $\text{ERR}\alpha$  orphan nuclear receptor gene into the pronucleus of a zygote derived from the animal of a), the zygote containing a homozygous disruption of the endogenous  $\text{ERR}\alpha$  orphan nuclear receptor gene;

15 (c) transplanting the animal zygote into a pseudopregnant compatible animal;

(d) allowing the zygote to develop to term;

(e) obtaining a founder animal carrying the transgene; and

20 (f) breeding the founder animal with a wild-type animal to obtain progeny that express the non endogenous  $\text{ERR}\alpha$  orphan nuclear receptor transgene at levels sufficient to functionally complement the disrupted  $\text{ERR}\alpha$  receptor activity.

Further, in accordance with the present invention, there 25 is also provided a method for screening and identifying a compound which modulates  $\text{ERR}\alpha$  orphan nuclear receptor activity. The method includes:

a) exposing the non-human transgenic animal in accordance with the present invention to a candidate compound, and;

5 b) determining the activity of the ERR $\alpha$  orphan nuclear receptor in the animal, where an increase in the receptor activity as compared to an unexposed non-human animal is indicative of a compound being capable of increasing ERR $\alpha$  orphan nuclear receptor activity, while a decrease in the receptor activity as compared to an unexposed non-human animal, is indicative of a compound being capable of decreasing ERR $\alpha$  orphan nuclear receptor activity.

10 Similarly, in accordance with the present invention, there is also provided a method of identifying an agent which modulates fat and/or glucose metabolism *in vivo* which comprises:

15 a) administering an agent suspected of being a modulator of ERR $\alpha$  activity and/or level in an animal;  
b) measuring lipid and/or glucose levels in the animal of step a) and comparing same with that of a control animal not having been administered the agent, wherein a difference in lipid and/or glucose levels of the animal of step a) as compared to that of the control animal, identifies the agent as a modulator of fat and/or glucose metabolism *in*  
20 *vivo*.

As well, there is also provided a method of identifying an agent which modulates fat and/or glucose metabolism *in vivo* which comprises:

25 a) providing a promoter operably linked to a selectable or assayable marker, the promoter being modulated by ERR $\alpha$ ;  
b) measuring or selecting for the marker in a presence and in an absence of an agent suspected of modulating the promoter modulating activity of ERR $\alpha$ , thereby identifying an agent which

modulates  $ERR\alpha$  activity wherein a difference in the transcriptional activity in the presence of the agent, as compared to that in the absence thereof, identifies the agent as a modulator of  $ERR\alpha$  activity:

- c) administering the agent identified in b) to a non-human transgenic animal according to the present invention; and
- 5 d) measuring lipid and/or glucose levels in the animal of step c) and comparing same with that of a control animal, not having been administered the agent, wherein a difference in lipid and/or glucose levels of the animal of step c) as compared to that of the control animal
- 10 identifies the agent as a modulator of fat and/or glucose metabolism *in vivo*.

Furthermore, in accordance with the present invention, there is provided a modulator of fat and/or glucose metabolism *in vivo* identified by a method of the present invention.

15 In accordance with the present invention, there is also provided a method of modulating fat tissue growth and/or weight gain. The method comprises administering to an animal an agent which modulates the promoter activity of a gene, wherein the promoter comprises *cis*-acting elements selected from the group consisting of:

- 20 i) an estrogen response element;
- ii) TGA AGG TCA;
- iii) AGG TCA NNN TGA CCT; and
- iv) functional variants of i-iii)

such as to modulate the level of the gene, thereby modulating fat tissue growth and/or weight gain in the animal.

25 In accordance with another embodiment of the present invention, there is provided a method of determining whether an agent modulates fat tissue growth and/or weight gain in an animal comprising:

- a) providing a transcriptionally active preparation of ERR $\alpha$  or related factors and a DNA sequence comprising a promoter having a cis-acting sequence which modulates activity thereof by an interaction thereto of said ERR $\alpha$  and related factors;
- 5 b) measuring the transcriptional activity of the promoter or of a binding of at least ERR $\alpha$  or related factors to the cis-acting sequence in a presence and in an absence of an agent suspected of modulating the transcriptional activity of the promoter or the binding of the factors to the cis-acting sequence, thereby identifying an agent which modulates transcription of the promoter and wherein a difference in the transcriptional activity and/or binding in the presence of the agent, as compared to that in the absence thereof identifies the agent as a modulator of transcription;
- 10 c) administering the agent identified in b) to a non-human transgenic animal according to one of claims 1 to 7; and
- 15 d) measuring fat tissue growth and/or weight gain in the animal of step c) and comparing same with that of a control animal, not having been administered the agent, wherein a difference in fat tissue growth and/or weight gain of the animal of step c) as compared to that of the control animal identifies the agent as a modulator of fat tissue growth and/or weight gain *in vivo*.

20 In accordance with yet another embodiment of the present invention, there is provided a method of treating and/or preventing obesity, comprising administering to an obese animal, or an animal susceptible of becoming obese, an agent which modulates the promoter activity of a promoter comprising a cis-acting element selected from the group consisting of:

- i) an estrogen response element;

- iii) TGA AGG TCA;
- iii) AGG TCA NNN TGA CCT; and
- iv) functional variants of i-iii)

wherein the cis-acting element is capable of binding to ERR $\alpha$ .

5 And yet in accordance with a further embodiment of the present invention, there is provided a method of determining whether an agent modulates obesity in an animal comprising:

10 a) providing a transcriptionally active preparation of ERR $\alpha$  or related factors and a DNA sequence comprising a promoter having a cis-acting sequence which modulates activity thereof by an interaction thereto of the ERR $\alpha$  and related factors;

15 b) measuring the transcriptional activity of the promoter or of a binding of at least ERR $\alpha$  or related factors to the cis-acting sequence in a presence and in an absence of an agent suspected of modulating the transcriptional activity of the promoter or the binding of the factors to the cis-acting sequence, thereby identifying an agent which modulates transcription of the promoter and wherein a difference in the transcriptional activity and/or binding in the presence of the agent, as compared to that in the absence thereof identifies the agent as a

20 modulator of transcription;

c) administering the agent identified in b) to a non-human transgenic animal according to one of claims 1 to 7; and

25 d) assessing obesity in the animal of step c) and comparing same with that of a control animal, not having been administered the agent, wherein a difference in obesity of the animal of step c) as compared to that of the control animal identifies the agent as a modulator of obesity *in vivo*.

For the purpose of the present invention, the following abbreviations and terms are defined below.

### **DEFINITIONS**

5 As used herein, the terminology "transgenic animal" refers to any animal which harbors a nucleic acid sequence having been inserted into a cell and having become part of the genome of the animal that develops from that cell. In a preferred embodiment, the transgenic animal is a mammal, in an especially preferred embodiment, the transgenic mammal is a mouse. However, other transgenic animals are 10 encompassed as within scope of the present invention. Non-limiting examples of such transgenic animals include transgenic rodents (i.e. rats, hamsters, guinea pigs, and rabbits), and transgenic pigs, cattle and sheep, as well as transgenic poultry. Techniques for the preparation of 15 such transgenic animals are well known in the art (e.g. introducing a transgene in ES cells; microinjecting the transgene into the male pronucleus of a fertilized egg; or infecting a cell with a recombinant virus). Indeed, lean transgenic animals may find utility in the food industry, in view of the increasing awareness of consumers to the degree of fat in 20 meat products.

As used herein, "non-human transgenic animal" is any non-human animal in which at least one cell comprises genetically altered information through known means such as microinjection, virus-delivered infection, or homologous recombination. In one particularly preferred 25 embodiment of the present invention, the transgenic animal is a transgenic mouse, in which the genetic alteration has been introduced in a germ-line cell such that it enables the transfer of this genetic alteration

to the offsprings thereof. Such offsprings containing this genetic alteration, are also transgenic mice.

The terminology "gene knockout" or "knockout" refers to a disruption of a nucleic acid sequence which significantly reduces and 5 preferably suppresses or destroys the biological activity of the polypeptide encoded thereby. For example, ERR $\alpha$  knockout animal refers to an animal in which the expression of ERR $\alpha$  has been reduced or suppressed by the introduction of a recombinant nucleic acid molecule comprising 10 ERR $\alpha$  sequences that disrupt at least a portion of the genomic DNA sequence encoding ERR $\alpha$  in the animal. A knockout animal might have one or both copies of the preselected nucleic acid sequence disrupted. In the latter case, in which a homozygous disruption is present, the 15 mutation is termed a "null" mutation. In a case where only one copy of a preselected nucleic acid sequence is disrupted, the knockout animal is a "heterozygous knockout animal".

The terminology "estrogen response elements" or "estrogen cis-acting elements" refers to well-known nucleic acid sequences to which transcription factors such as the orphan nuclear receptor ERR $\alpha$  can bind, thereby having the potential to modulate the 20 promoter activity of a promoter comprising such response or cis-acting elements. These cis-acting elements or estrogen response elements also termed "ERE" or "IR3" are well-known in the art (Petterson, 1996, *Mech. Dev.* 54:211-223). In Petterson et al. (1996, *supra*), it is for example taught that the perfect inverted repeat (IR) of the estrogen response 25 element to which ERR $\alpha$  can bind has sequence AGG TCA NNN TGA CCT. It is also known from Sladek et al., 1997, Bonnelye et al., 1997 and Johnston et al., 1997 that this acting element comprising the sequence TGA AGG TCA can also bind ERR $\alpha$  and related factors.

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, 5 infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

10 Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

15 The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

20 "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (i.e. genomic DNA, cDNA) and RNA molecules (i.e. mRNA). The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

25 The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred 5 to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, 10 preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected 15 conditions.

The nucleic acid (i.e. DNA or RNA) for practising the present invention may be obtained according to well known methods.

As used herein, the term "physiologically relevant" is meant to describe the functional relevance of a nucleic acid and/or protein 20 in its natural setting.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 12 25 nucleotides in length, preferably between 15 and 24 nucleotides, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the

melting point of hybridization thereof with its targeted sequence (see below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

5                   The term "oligonucleotide" or "DNA" molecule or sequence refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as the term is defined herein. The 10 term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

15                   " Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favoured double-stranded structure. Examples 20 of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989, *supra*) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labelled probe in a solution containing 50% formamide, high 25 salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in

view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such 5 cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al., 1989, *supra*).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones 10 including phosphorothioates, dithionates, alkyl phosphonates and  $\alpha$ -nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or 15 deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labelled proteins could also be used to detect a particular nucleic acid 20 sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like. Of course, it will be understood that the present invention lends itself to semi- or full- automated screening techniques. A non limiting of such a screening technique includes the known gene chips technology.

25 Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes

can be labelled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ , and  $^{35}\text{S}$ . Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other 5 detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

10 As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma  $^{32}\text{P}$  ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labelled DNA 15 probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). 20 The size of the oligo will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide 25 which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted 5 to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Q $\beta$  replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et 10 al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 15 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one 20 oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products 25 using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization

following EtBr staining of the DNA following gel electrophoresis, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

5                   Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, *Science* 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the 10 particular needs (Walker et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:392-396; and *ibid.*, 1992, *Nucleic Acids Res.* 20:1691-1696).

15                   As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

20                   A "heterologous" (i.e. a heterologous gene) region of a DNA molecule is a subsegment segment of DNA within a larger segment that is not found in association therewith in nature. The term "heterologous" can be similarly used to define two polypeptidic segments not joined together in nature. Non-limiting examples of heterologous 25 genes include reporter genes such as luciferase, chloramphenicol acetyl transferase,  $\beta$ -galactosidase, and the like which can be juxtaposed or joined to heterologous control regions or to heterologous polypeptides.

The terminology "endogenous gene" generally defines the gene which has been disrupted to produce the knockout transgenic animal. In a particular embodiment relating to a knockout mice, the endogenous  $ERR\alpha$  gene is the mouse  $ERR\alpha$  gene. In a related aspect, 5 the terminology "non-endogenous transgene" should be generally understood as a transgene which is not in its natural setting (e.g. different expression control elements), was isolated from a different species (e.g. human), or has been engineered to display a new characteristic (e.g. an engineered mutant gene)

10 The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

15 The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), and the mRNA translated (translation) into one polypeptide (or protein) or more.

20 The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

25 Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is

not necessary that two sequences be immediately adjacent to one another.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a 5 prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites. In addition, the expression control sequence can confer constitutive or inducible expression upon the 10 sequence to which it is operably linked.

Prokaryotic expressions are useful for the preparation of large quantities of the protein encoded by the DNA sequence of interest. This protein can be purified according to standard protocols that take advantage of the intrinsic properties thereof, such as size and 15 charge (i.e. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal antibodies. Polyclonal antibodies which can be used in the context of the present invention have been described (Sladek et al. *Supra*). The purified 20 protein can be used for therapeutic applications.

The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" 25 refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and

extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as 5 protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

10 As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether an nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent 15 may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or 20 additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid as chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, 25 hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

5 The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. all these methods are well known in the art.

10 As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in Remington's Pharmaceutical Sciences (e.g. 1980). Methods of coupling these chemical-physical moieties to a polypeptide are well known in the art.

15 The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

20 As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

25 As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A

'substantially pure' molecule is a molecule that is lacking in all other cellular components.

As used herein, the terms "molecule", "compound", "agent" or "ligand" are used interchangeably and broadly to refer to 5 natural, synthetic or semi-synthetic molecules or compounds. The term "molecule" therefore denotes for example chemicals, macromolecules, cell or tissue extracts (from plants or animals) and the like. Non limiting examples of molecules include nucleic acid molecules, peptides, antibodies, carbohydrates and pharmaceutical agents. The agents can 10 be selected and screened by a variety of means including random screening, rational selection and by rational design using for example protein or ligand modelling methods such as computer modelling. The terms "rationally selected" or "rationally designed" are meant to define compounds which have been chosen based on the configuration of the 15 interaction domains of the present invention. As will be understood by the person of ordinary skill, macromolecules having non-naturally occurring modifications are also within the scope of the term "molecule". For example, peptidomimetics, well known in the pharmaceutical industry and generally referred to as peptide analogs can be generated by modelling 20 as mentioned above. Similarly, in a preferred embodiment, the polypeptides of the present invention are modified to enhance their stability. It should be understood that in most cases this modification should not alter the biological activity of the interaction domain. The molecules identified in accordance with the teachings of the present 25 invention have a therapeutic value in diseases or conditions in which the physiology or homeostasis of the cell and/or tissue is compromised by a defect in ERR $\alpha$  or in pathways converging thereon or therefrom. Alternatively, the molecules identified in accordance with the teachings

of the present invention find utility in the development of more efficient agents which modulate ERR $\alpha$  level or activity. In a preferred embodiment, the molecules are agonists and antagonists compounds of ERR $\alpha$  activity. Such compounds can be steroid-like or non-steroidal 5 compounds. Of course, these compounds can be identified from libraries (e.g. a combinatorial library). Since the ERR $\alpha$  receptor is phosphorylated *in vivo*, compounds which modulate ERR $\alpha$  receptor activity through phosphorylation could also be identified. The compounds identified in accordance with the present invention could be modified as known by the 10 person of ordinary skill so as to target a chosen or specific tissue- or cell-type.

In one embodiment, agonists or antagonists of ERR $\alpha$  can be detected and selected by contacting the indicator cell or animal with a compound or mixture or library of molecules for a fixed period of 15 time and an activity of ERR $\alpha$  is then determined.

In one particular embodiment, the level of gene expression of ERR $\alpha$  can be determined directly or indirectly (e.g. through the level of a reporter gene such as luciferase, or  $\beta$ -gal) within the treated cells or animal and compared to the level thereof in the absence of the 20 molecules(s). The difference between the levels of gene expression indicates whether the molecule(s) agonizes or antagonizes the expression of ERR $\alpha$ . The magnitude of the level of the effect of the molecule(s) (treated vs. untreated cells) provides a relative indication of the strength of that molecule(s). The same type of approach can also be 25 used in the presence of an antagonist(s).

As well, having identified ERR $\alpha$  as a target for lipogenesis, fatty acid esterification and fatty acid oxydation modulation. ERR $\alpha$  can be used in a number of *in vitro* and *in vivo* assays to

identify ligands therefor and dissect its structure/function relationship. Non limiting examples thereof include binding assays and the two hybrid system technology, as well known in the art (Ausubel et al., 1994, *supra*). This assay has proven beneficial to test compounds or a library thereof.

5 Thus, the invention also covers ERR $\alpha$ -expressing cells (prokaryotes, lower and higher eukaryotes) or variants thereof to identify mutations which modulate ERR $\alpha$  activity or compounds which have ERR $\alpha$  modulating effects.

The present invention also provides antisense nucleic acid molecules which can be used for example to decrease or abrogate the expression of the nucleic acid sequences or proteins of the present invention. An antisense nucleic acid molecule according to the present invention refers to a molecule capable of forming a stable duplex or triplex with a portion of its targeted nucleic acid sequence (DNA or RNA). The 10 use of antisense nucleic acid molecules and the design and modification of such molecules is well known in the art as described for example in WO 96/32966, WO 96/11266, WO 94/15646, WO 93/08845 and USP 5,593,974. Antisense nucleic acid molecules according to the present invention can be derived from the nucleic acid sequences and 15 modified in accordance to well known methods. For example, some antisense molecules can be designed to be more resistant to degradation to increase their affinity to their targeted sequence, to affect their transport to chosen cell types or cell compartments, and/or to enhance their lipid solubility by using nucleotide analogs and/or substituting 20 chosen chemical fragments thereof, as commonly known in the art. 25

Of course, the cells or animals in accordance with the present invention can be used to identify antagonists.

A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting DNA may be maintained on a episomal element such as a plasmid. With respect to eukaryotic cells, a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA. Transfection methods are well known in the art (Sambrook et al., 1989, *supra*; Ausubel et al., 1994 *supra*). The use of a mammalian cell as indicator can provide the advantage of furnishing an intermediate factor, which permits for example the interaction of two polypeptides which are tested, that might not be present in lower eukaryotes or prokaryotes. Of course, an advantage might be rendered moot if two polypeptides or interacting domains thereof are tested. It will be understood that extracts from mammalian cells for example could be used in certain embodiments, to compensate for the lack of certain factors in a chosen indicator cell.

An indicator cell in accordance with the present invention can be used to identify antagonists. For example, the test molecule or molecules are incubated with the host cell in conjunction with one or more agonists held at a fixed concentration. An indication and relative strength of the antagonistic properties of the molecule(s) can be provided by comparing the level of gene expression in the indicator cell in the presence of the agonist, in the absence of test molecules versus in

the presence thereof. Of course, the antagonistic effect of a molecule could also be determined in the absence of agonist, simply by comparing the level of expression of the reporter gene product in the presence and absence of the test molecule(s).

5 It shall be understood that the "in vivo" experimental model can also be used to carry out an "in vitro" assay. For example, cellular extracts from the indicator cells and/or cellular extracts from the non-human transgenic animals of the present invention can be prepared and used in one of the *in vitro* method of the present invention or an *in*  
10 *vitro* method known in the art. Non-limiting examples of such assays are taught in U.S.P. 5,298,429. It should be noted that U.S.P. 5,298,429 also teaches the sequence of ERR $\alpha$  and ERR $\beta$  from human, as well as the significant conservation in the sequence of ERR $\alpha$ , ERR $\beta$ , and related family members.

15 In one particular embodiment, an "indicator cell" can be designed so as to express ERR $\alpha$  so as to modulate a promoter operably linked to a reporter gene, or to an identifiable or selectable phenotype or characteristic such that it provides an assessment of the activity and/or level of ERR $\alpha$ . Such indicator cells can be used in the screening assays  
20 of the present invention. In certain embodiments, the indicator cells have been engineered so as to express a chosen derivative, fragment, homolog, or mutant of ERR $\alpha$ . The cells can be prokaryotic cells, yeast cells or higher eukaryotic cells such as mammalian cells (WO 96/41169). In one particular embodiment, the indicator cell is a yeast cell harboring  
25 vectors enabling the use of the two hybrid system technology, as well known in the art (Ausubel et al., 1994, *supra*) and can be used to test a compound or a library thereof. In one embodiment, a reporter gene encoding a selectable marker or an assayable protein can be operably

linked to a control element such that expression of the selectable marker or assayable protein is dependent on the interaction of ERR $\alpha$  with an interacting protein thereof. Such an indicator cell could be used to rapidly screen at high-throughput a vast array of test molecules. In a particular 5 embodiment, the reporter gene is luciferase or  $\beta$ -Gal.

In one embodiment, at least one of the ERR $\alpha$  and a protein or domain thereof with which it interacts may be provided as a fusion protein. The design of constructs therefor and the expression and production of fusion proteins are well known in the art (Sambrook et al.. 10 1989, *supra*; and Ausubel et al., 1994, *supra*). In a particular embodiment, both interaction domains are part of fusion proteins.

Non limiting examples of such fusion proteins include a hemagglutinin fusions, Gluthione-S-transferase (GST) fusions and Maltose binding protein (MBP) fusions. In certain embodiments, it might be 15 beneficial to introduce a protease cleavage site between the two polypeptide sequences which have been fused. Such protease cleavage sites between two heterologously fused polypeptides are well known in the art.

In certain embodiments, it might also be beneficial to 20 fuse the interaction domains of the present invention to signal peptide sequences enabling a secretion of the fusion protein from the host cell. Signal peptides from diverse organisms are well known in the art. Bacterial OmpA and yeast Suc2 are two non limiting examples of proteins containing signal sequences. In certain embodiments, it might also be 25 beneficial to introduce a linker (commonly known) between the interaction domain and the heterologous polypeptide portion. Such fusion protein find utility in the assays of the present invention as well as for purification purposes, detection purposes and the like.

For certainty, the sequences and polypeptides useful to practice the invention include without being limited thereto mutants, homologs, subtypes, alleles and the like. It shall be understood that generally, the sequences of the present invention should encode a 5 functional (albeit defective) interaction domain. It will be clear to the person of ordinary skill that whether an interaction domain of the present invention, variant, derivative, or fragment thereof retains its function in binding to its partner or in modulating transcription can be readily determined by using the teachings and assays of the present invention 10 and the general teachings of the art.

In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in 15 Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody- A Laboratory Manual, CSH Laboratories). The present invention also provides polyclonal, monoclonal antibodies, or humanized versions thereof, chimeric antibodies and the like which inhibit or neutralize their 20 respective interaction domains and/or are specific thereto.

From the specification and appended claims, the term therapeutic agent should be taken in a broad sense so as to also include a combination of at least two such therapeutic agents. Further, the DNA segments or proteins according to the present invention can be 25 introduced into individuals in a number of ways. For example, a chosen cell type cell can be isolated from the afflicted individual, transformed with a DNA construct according to the invention and reintroduced to the afflicted individual in a number of ways, including intravenous injection.

Alternatively, the DNA construct can be administered directly to the afflicted individual, for example, by injection in the bone marrow. The DNA construct can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, 5 and engineered to be administered through different routes.

In one particular embodiment, the present invention provides the means to treat weight-related diseases or conditions comprising a decrease or total eradication of ER $\alpha$  expression. It will be recognized that having shown that the absence of ER $\alpha$  expression 10 reduces fat tissue, provides numerous means of achieving fat reduction in animals.

For administration to humans, the prescribing medical professional will ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen 15 therapeutic regimen (i.e. DNA construct, protein, cells), the response and condition of the patient as well as the severity of the disease.

Composition within the scope of the present invention should contain the active agent (i.e. fusion protein, nucleic acid, and molecule) in an amount effective to achieve the desired therapeutic effect 20 while avoiding adverse side effects. Typically, the nucleic acids in accordance with the present invention can be administered to mammals (i.e. humans) in doses ranging from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the 25 present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will

be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.1ng to 1g/kg/day, and preferably 10 mg to 50 mg/kg/day will be administered to the mammal.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

10 Figure 1 shows the targeted disruption of the *Estrra* gene and heterozygote inbreeding analysis. a. Structure of the ERR $\alpha$  locus, targeting vector, and recombinant allele. Top, map of the wild type locus: exons are indicated by black boxes. E2 encodes the upstream zinc-binding motif of the ERR $\alpha$  DNA-binding domain. Center, targeting construct. Bottom, map of the targeted allele, showing replacement of exon 2 sequences by the *neo'* cassette. The restriction enzyme digests and the probes used to characterize the knockout mice are illustrated. B. *BamHI*; H, *HindIII*. b. Southern blot analysis of targeted ES clones. DNA from parental ES cells (R1) and two targeted clones (57 and 62) was digested with *BamHI* and hybridized to the 3' probe. The positions of bands corresponding to the wild-type (10.7 kb) and targeted alleles (4.5 kb) are indicated (upper panel). Single integration of the targeting construct in targeted ES cell clones was confirmed with a *neo'* probe: a single hybridizing band (6.0 kb) is present in the targeted lines (lower panel). c, Southern blot analysis of genotypes of 28d old pups from a heterozygote intercross: the litter contains viable homozygous null mice. d, Northern blot analysis of RNA obtained from the kidneys of the progeny

of heterozygous intercrosses. ERR $\alpha$  expression is not detected in RNA samples obtained from homozygous null mutants.

Figure 2 shows the phenotypic analysis of *Estrra* null mutants. *a*, Mutant animals display decreased weight gain. Growth curves were performed by weighing animals at the indicated ages: both male and female knockout mice display significantly reduced body weight in comparison to their wild-type littermates. Arrows indicate start of pre-pubertal growth spurt. *b*, Body composition of *Estrra* null mice shows decreased ratio of fat to lean mass. *c*, *Estrra*<sup>-/-</sup> mice contain decreased body fat. Superficial carcass dissection of two 20 week old male mice shows the decreased body fat content of a 32.9 g knockout mouse (right) in comparison with his 38.1 g wild-type littermate (left). *d*, The difference in body composition is reflected by the relative sizes of the dissected fat pads.

Figure 3 shows the analysis of intestinal lipid transport in *Estrra* null mutants. *a*, Thin layer chromatographic analysis of tissue lipid content. The intestines of *Estrra*<sup>-/-</sup> mice contain decreased triglyceride and increased free fatty acids in comparison with their wild-type and heterozygous littermates. *b*, Analysis of glycerolipid synthesis in *Estrra* null mutants. *Estrra*<sup>-/-</sup> mice demonstrate reduced triglyceride synthesis in intestinal and hepatic whole cell extracts. *c*, Fat absorption profile. *Estrra*<sup>-/-</sup> mice and littermate controls display similar rates of absorption of radiolabeled oleic acid.

Figure 4 shows the analysis of adipocyte function in *Estrra* null mutants. *a*, Histologic studies of epididymal fat pads show that *Estrra*<sup>-/-</sup> mice (lower panel) have decreased adipocyte volume in comparison to wild-type animals (upper panel). *b*, *Estrra*<sup>-/-</sup> mice demonstrate decreased lipogenesis in comparison to littermate controls.

Following intraperitoneal injection with  $^3\text{H}_2\text{O}$ , *Estrra* mice incorporate 30-55% less  $^3\text{H}$  into adipose tissue and 50% less  $^3\text{H}$  into hepatic lipids. IF, inguinal fat; EF, epididymal fat; PF, perirenal fat.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

10 **DESCRIPTION OF THE PREFERRED EMBODIMENT**

The method of production and the transgenic animals of the present invention are described herein below. In general, these animals are produced by engineering a nucleic acid construct which can disrupt the expression of the endogenous  $\text{ERR}\alpha$  gene (i.e., the murine  $\text{ERR}\alpha$  gene). Using known methods, this construct is amplified in bacterial cells, purified, and transferred into ES cells or isolated oocytes. The transfected ES cells can then be injected into blastocysts to generate chimeras. The chimeras which transmit the mutation to their offspring are identified and selected. These animals can then be used as founder animals to obtain different animal lines, derived from breeding with chosen animals. Heterozygous animals can then be produced and further mated to generate a hybrid F1 cross. Further matings of the F1 heterozygotes produce the wild type, heterozygous and homozygous null mutants of  $\text{ERR}\alpha$  (having both copies of the  $\text{ERR}\alpha$  gene disrupted). The homozygous animals can then serve in a number of experiments. Non-limiting examples thereof include : the characterization of their phenotype, and a reconstitution of the  $\text{ERR}\alpha$  activity by complementation by a non-endogenous copy of a wild type  $\text{ERR}\alpha$  gene or mutant or variant  $\text{ERR}\alpha$ .

gene. An animal (or cells derived therefrom) expressing a mutant form of ERR $\alpha$  gene (from human, for example) could be used to screen for compounds which modulate more specifically the mutant form of the ERR $\alpha$  gene.

5 The present invention therefore strongly indicates that ERR $\alpha$  is a direct regulator of fundamental cellular function. It is thus expected that this cellular function should occur across species. The presence of the ERR $\alpha$  gene and its conservation among species (human, mice, rats, fish and lower organisms; Escriva et al. (1997) Proc. Natl. Acad. Sci. USA 94:6803-6808), support its essential role in physiology. 10 Thus, the antagonists identified by the methods and assays of the present invention should find a utility in the treatment of obesity and other metabolic diseases associated with ERR $\alpha$  malfunction in humans.

15 The present invention is illustrated in further detail by the following non-limiting examples.

### EXAMPLE 1

#### **Creation of *Estrra*<sup>-/-</sup> mice**

20 Three overlapping  $\lambda$  clones containing the mouse *Estrra* locus were isolated from a 129Sv genomic library (gift of Dr. A. Joyner, Skirball Institute, New York) and characterized by restriction mapping and direct sequencing of the exon boundaries. The knockout construct was created using pNT (Tybulewicz et al., 1991) and contained 6.4 kb of genomic DNA flanking the second exon of *Estrra*. An endfilled 4.2 Kb *Bam*H/I/*Not*I fragment, lying upstream of the second exon, was cloned into the *Xba*I site of pNT, while a 2.2 Kb *Hind*III fragment was cloned between the *neo*' and TK cassettes to provide the 3' arm of the construct. Correct targeting of the *Estrra* locus replaces the receptor's second exon, which 25

encodes a critical part of its DNA binding domain, with a *neo* cassette. The linearized construct was electroporated into R1 ES cells (Nagy and Rossant, 1993) which were selected with G418 (150 µg/mL) and gancyclovir (2 µM). Two ES cell clones were isolated and injected into 5 C57BL6 blastocysts to generate chimeras, and three chimeras transmitted the mutation to their offspring. Heterozygous mice, generated by mating the chimeric animals with 129SvJ mice were mated with C57BL6 animals to generate hybrid F1 animals; physiologic studies were performed using the F2 null mutant and wild-type offspring obtained by 10 mating the F1 hybrid heterozygotes. Complete disruption of the *Estrra* allele was verified by performing Northern blots using RNA obtained from placenta and kidneys of homozygous mutants.

#### EXAMPLE 2

15 **Physiological parameters of *Estrra*<sup>-/-</sup> mice**

Mice were housed in an SPF facility with a daily 12 h light cycle (7:00 to 19:00h) and with free access to food and water. Between two and four mice were contained in each cage. Growth curves were obtained by weighing mice of defined ages between 10:00 and 20 12:00h. Fasting serum and biochemical studies were performed between 10:00 to 12:00h using animals that had been deprived of food for 18 hrs. Body composition was determined by desiccating mouse carcasses from which the intestines had been removed. Following desiccation, the carcass was homogenized and a 1 g aliquot was saponified using potassium hydroxide and extracted with petroleum ether. Following complete evaporation of the ether, the residue was weighed to determine fat content. Rectal temperature was measured using a rectal probe in 25 animals housed at 29°C and 4 C. Baseline biochemical studies were

performed using serum samples obtained from tail bleeds of restrained animals at between 20 and 28 weeks of age. Enzymatic assays were used to determine serum triglycerides (GPO-PAP, Boehringer-Mannheim) and glycerol (TC Glycerin, Boehringer-Mannheim), glucose (Glucose Oxidase-Trinder, Sigma), free fatty acids (GPO-PAP Half Micro Test, Boehringer-Mannheim), and  $\beta$ -hydroxybutyrate (TC  $\beta$ -hydroxybutyrate, Boehringer-Mannheim).

### EXAMPLE 3

#### 10        **Organ lipid content and esterification rates of *Estrra*<sup>-/-</sup> mice**

Mice were allowed free access to food and water overnight. Experiments were performed between 09:00 and 11:00h, at which time the animals were sacrificed by cervical dislocation and their tissues harvested and frozen in liquid nitrogen. To study tissue lipid content, the frozen tissues were pulverized on a precooled anvil and homogenized in cold 1 x PBS. The homogenate was extracted using a 4:1 volume ratio of Folch buffer (chloroform: methanol). The extracted lipids were separated by thin-layer chromatography using a silica plate (Whatman LK5D) and visualized by iodine staining. Intestinal fatty acid esterification was studied using pulverized tissue, which was homogenized briefly in 1 x PBS. Following brief centrifugation (13,000 rpm, 5 min, 4°C) to pellet cell nuclei and membrane debris, the soluble protein fraction was extracted and quantified using the Bradford reagent. Between 50 and 200 micrograms of crude protein extract was used to study the incorporation of {9,10-<sup>3</sup>H}oleic acid (New England Nuclear) into glycerolipids using to previously published methods (Yasruel et al., 1991).

#### EXAMPLE 4

##### **Measurement of a physiology parameter of the transgenic mouse: lipogenesis rate measurements of *Estrra*<sup>-/-</sup> mice**

Mice were studied at 10:00h following free access to food overnight. The animals were conditioned by sham intraperitoneal injections of water. On the day of the experiment, the animals were injected intraperitoneally with  $^3\text{H}_2\text{O}$  (0.5 mCi per 100 g body weight) and sacrificed by cervical dislocation 30 minutes later. Serum, adipose tissue and liver samples were harvested and stored at -80°C. The tissues were homogenized and heated in ethanolic KOH: the resulting extract, which contained saponified lipids, was acidified using concentrated sulfuric acid and extracted using petroleum ether. The extract was dried by evaporation and  $^3\text{H}$  incorporation determined by scintillation counting.

The *Estrra* gene was inactivated in embryonic stem (ES) cells using a targeting vector which replaces exon 2 of the receptor with the *neo'* gene: this exon encodes a critical portion of the receptor's DNA binding domain (Fig. 1A). Two correctly targeted ES cell clone were obtained (Fig. 1B), one of which (clone #62) was injected into C57BL/6 blastocysts to generate chimeric animals. Three chimera transmitted the targeted allele to their offspring. Heterozygous mice were generated by mating the founder animals with 129/SvJ mice which were then mated with C57BL/6 animals to generate an hybrid F1 cross. Litters obtained from mating the F1 heterozygotes contained appropriate numbers of wild type, heterozygous and homozygous null animals (Fig. 1C). In addition, ER $\alpha$  null mutants underwent grossly normal intrauterine development, were fertile, appeared healthy and did not exhibit increased mortality when compared to their wild-type littermates. Northern blot analysis of RNA obtained from the kidneys of homozygous mutants confirmed

complete disruption of the *Estrra* locus. no ERR $\alpha$  transcripts were detected in tissues obtained from homozygous null mutants (Fig. 1D).

Phenotypic analysis of embryonic and post-natal mice was performed using F2 hybrid strain animals. Male and female *Estrra*<sup>-/-</sup> mutants displayed significantly decreased body mass, which was not associated with changes in body length or in the time of onset of pre-pubertal growth (Table 1 and Fig. 2A). Body composition studies were performed using male animals, and revealed that ERR $\alpha$  null mutants contained 32% less body fat and a decrease in fat to lean mass ratio (Table 1 and Fig. 2B), and decreased white adipose tissue (WAT) deposits (Fig. 2C and D). Decreased food intake or increased fat excretion (Table 1) could not account for this alteration in body composition. In addition, fasting serum triglyceride and serum glucose levels were identical in wild-type and knockout animals, demonstrating that the mutant animals had normal hepatic synthetic function (Table 2).

**TABLE 1**  
**Physiological characterization of male *ERR $\alpha$*  mice**

Genotype	BW	Length (cm)	Fat (g)	Lean mass (g)	Water (g)	Food Intake (g/d)	Fecal Fat (%)
Control (+/+, +/−)	32.7 $\pm$ 1.0	9.6 $\pm$ 0.1	6.89 $\pm$ 0.70	6.86 $\pm$ 0.15	14.8 $\pm$ 0.22	5.17 $\pm$ 0.21	3.03 $\pm$ 0.22
Mutant (−/−)	27.3 $\pm$ 1.0	9.3 $\pm$ 0.4	4.64 $\pm$ 0.53	6.03 $\pm$ 0.18	13.1 $\pm$ 0.41	4.93 $\pm$ 0.27	3.03 $\pm$ 0.08
% of control	83.4%	96.8%	67.3%	88.0%	88.6%	95.3%	100.0%
p < 0.001	NS	p < 0.02	p < 0.01	p < 0.01	NS	NS	—

**TABLE 2**  
**Biochemical characterization of male ERR $\alpha$  mice**

Genotype	Fasting BG (mg/dL)	Fasting TG (mg/dL)	Fasting FFA ( $\mu$ m)
Control ( $+/+$ )	111.0 $\pm$ 8.2	108.8 $\pm$ 8.7	596 $\pm$ 41
Mutant ( $-/-$ )	107.0 $\pm$ 7.6	107.2 $\pm$ 4.9	751 $\pm$ 72
% of control	96.3%	98.5%	126%
	NS	NS	NS

In order to further characterize the mechanism causing decreased fat mass in ERR $\alpha$  knockout mice, a determination as to whether these animals had subtle defects in intestinal triglyceride absorption was sought. TLC analysis of lipids obtained from whole cell extracts showed that the intestines of ERR $\alpha$  null mice contained increased levels of free fatty acids and decreased levels of triglycerides in comparison with wild type mice (Fig. 3A). This observation suggests that ERR $\alpha$  mice have decreased intestinal capacity for fatty acid esterification, a hypothesis that is confirmed by *in vitro* measurement of glycerolipid synthesis in intestinal whole cell extracts (Fig. 3B). Decreased intestinal fatty acid activation or esterification capacity would be expected to delay the rate at which dietary fatty acids and triglycerides are

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transferred across the intestine or to reduce the maximum serum triglyceride levels observed following a fat load; however, assessment of intestinal oleic acid transport *in vivo* shows that ERR $\alpha$  knockout animals and wild-type mice have similar rates of fatty acid absorption (Fig. 3C).

5 Whether the abnormalities observed in intestinal lipid metabolism *in vitro* play major roles in the abnormal body composition observed in ERR $\alpha$  knockout mice is unclear; however, reduction of the maximal rate at which the intestines esterify dietary lipids may prevent *Estrra* $^{\text{-}}$  mice from increasing intestinal energy transfer in order to compensate for other 10 defects in fat or energy metabolism.

Previous studies have demonstrated that ERR $\alpha$  is highly expressed in brown adipose tissue (BAT) during murine development and that the receptor is upregulated during white and brown adipocyte differentiation *in vitro* (Sladek et al., 1997; Vega and Kelly, 1997). In 15 addition, ERR $\alpha$  has been shown to modulate the activity of the medium chain acyl-coA dehydrogenase (MCAD) promoter, a key regulatory step in the fatty acid  $\beta$ -oxidation pathway (Sladek et al., 1997; Vega and Kelly, 1997). As dysregulation of BAT function has been associated with abnormalities of body composition, therefore, a characterization of BAT 20 function in the ERR $\alpha$  knockout mice was carried out. ERR $\alpha$  null mutants had normal core body temperature and basal metabolic rate and displayed normal expression levels of uncoupling protein (UCP) mRNA in BAT (UCP-1) and skeletal muscle (UCP-2) (data not shown). Defects in fatty acid oxidation are frequently only apparent following situations of 25 physiologic stress or food deprivation; neither prolonged cold exposure or fasts of up to 48 hour's duration resulted in any morbidity or mortality of *Estrra* $^{\text{-}}$  mice (data not shown). Taken together, these data suggest that the abnormal body composition seen in ERR $\alpha$  null mutants was not a

result of increased thermogenesis or increased basal energy expenditure, and that the animals did not have physiologically significant defects in fatty acid  $\beta$ -oxidation.

Fat pads obtained from *Estrra* mutants displayed decreased adipocyte volume in comparison to wild-type animals (Fig. 4A), which suggests that the decreased adipose tissue mass observed in *Estrra*<sup>-/-</sup> mice results from an imbalance between fatty acid synthesis and lipolysis rather than defects in adipocyte proliferation and differentiation. As ERR $\alpha$  expression is induced during early adipocyte differentiation *in vitro* (Sladek et al., 1997), it is possible that ERR $\alpha$  acts as a regulator of processes important for adipocyte function, such as fatty acid synthesis or esterification. In animals fed a standard laboratory diet, murine adipose tissue contains triglyceride formed from fatty acids that are synthesized *de novo* rather than from dietary lipid. Lipogenesis was assessed by treating *Estrra*<sup>-/-</sup> mice with  $^3\text{H}_2\text{O}$ : the amount of radioactive label incorporated into triacylglycerol can be measured by saponification and ether extraction of adipose tissues and other organs. *Estrra* null mutants demonstrate significantly decreased lipogenesis in comparison to littermate controls: in particular, knockout animals show a 30-55% decrease in  $^3\text{H}$  incorporation into adipose tissue lipids and a 50% decrease in  $^3\text{H}$  incorporation into hepatic lipids (Fig. 4B). This observation demonstrates that adipose tissue of knockout mice possesses a defect in TG synthesis, which may result from decreased adipocyte and hepatic glycolysis activity, fatty acid synthesis or esterification.

Experiments performed using the *Estrra*<sup>-/-</sup> mice revealed that ERR $\alpha$  is a key regulator of fat metabolism, including intestinal fat transfer and esterification, as well as hepatic and adipocyte fat deposition. *Estrra*<sup>-/-</sup> mice display decreased fat content associated with reduced

intestinal fatty acid esterification rates and abnormal regulation of fat deposition and mobilization in adipocytes and liver. Previous *in vitro* studies have demonstrated that ERR $\alpha$  modulates the expression of MCAD, a key regulatory enzyme of fatty acid  $\beta$ -oxidation, a pathway which may also play a role in establishing the ERR $\alpha$  phenotype. The relative importance of each of these effects in establishing the body composition of ERR $\alpha$  mice remains to be determined. Since the *Estrra*<sup>-/-</sup> mice show a normal level of energy intake, one would expect to observe an increase in energy expenditure to account for the decreased fat content of these mice. However, the sensitivity of fecal fat measurements and calorimetry experiments may not be sufficient to identify small differences between wild-type and knockout animals which over a period of time would be sufficient to explain the observed phenotype. Within these experimental limitations, the data presented herein demonstrate that ERR $\alpha$  mice are lean as a result of aberrant regulation of peripheral lipid mobilization. ERR $\alpha$  mice display an unique combination of properties that suggests that modulation of ERR $\alpha$  activity may provide an effective method to regulate fat metabolism and that ERR $\alpha$  would be a key drug target for the treatment of obesity and other disorders of fat deposition. In addition, the close linkage of ESTRRA and diabetes susceptibility locus IDDM4 (Sladek et al., 1997) together with physiological defects observed in *Estrra*<sup>-/-</sup> mice suggests that drugs influencing ERR $\alpha$  activity could also be used to treat diabetes and other metabolic disorders.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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**WHAT IS CLAIMED IS:**

1. A non-human transgenic animal whose germ cells and somatic cells contain a knockout mutation in the endogenous ERR $\alpha$  orphan nuclear receptor gene, and wherein said transgenic animal shows a phenotype of an altered fat and/or glucose metabolism as compared to a control animal.  
5
2. The transgenic animal of claim 1, wherein said germ cells and somatic cells contain a homozygous disruption of said ERR $\alpha$  gene, and wherein said disruption comprises the insertion of a selectable marker sequence.  
10
3. The non-human transgenic animal of claim 1 or 2, wherein said animal is a mammal.  
15
4. The non-human transgenic animal of claim 3, wherein said animal is a mouse.
- 20 5. The non-human transgenic animal of claims 1 to 4, displaying a lean phenotype.
- 25 6. The non-human transgenic animal of one of claims 1 to 5, whose germ cells and somatic cells additionally comprise a transgene encoding a non endogenous ERR $\alpha$  orphan nuclear receptor gene, wherein said transgene is expressed at levels sufficient to

complement the disrupted endogenous ERR $\alpha$  orphan nuclear receptor activity.

7 The non-human transgenic animal of claim 6,  
5 wherein said non endogenous ERR $\alpha$  orphan nuclear receptor gene is a  
human ERR $\alpha$  orphan nuclear receptor gene.

8 The non-human transgenic animal of claim 7,  
wherein said animal is a mouse and said non-endogenous ERR $\alpha$  is a  
10 human ERR $\alpha$  gene.

9. A cell line derived from the non-human transgenic  
animal of one of claims 1 to 8.

15 10. A method of producing a non-human transgenic  
animal, in which at least some cells thereof contain an altered gene  
encoding an altered ERR $\alpha$ , said altered gene having been targeted to  
disrupt the endogenous ERR $\alpha$  gene in said transgenic animal, said  
method comprising:

20 a) providing an altered gene encoding the altered form  
of ERR $\alpha$  and designed to target and disrupt said endogenous ERR $\alpha$   
gene of an embryonic stem cells (ES) of said animal;  
b) introducing said altered gene in said ES cells;  
c) selecting ES cells in which said altered ERR $\alpha$  gene  
25 has disrupted said endogenous ERR $\alpha$  gene;

- d) injecting said selected ES cells of c) into blastocysts;
- e) implanting said blastocysts of d) in a pseudopregnant animal; and
- 5 f) producing a non-human transgenic animal having at least some cells having said altered ERR $\alpha$  gene encoding said altered ERR $\alpha$ .

11. The method of claim 10, wherein said non-human  
10 transgenic animal is a mouse.

12. A method of producing the non-human transgenic animal of claim 5, said method comprising:

- (a) providing a non-human transgenic animal lacking detectable levels of ERR $\alpha$  orphan nuclear receptor gene and exhibiting a lean phenotype;
- 15 (b) introducing a non endogenous ERR $\alpha$  orphan nuclear receptor transgene encoding a functional ERR $\alpha$  orphan nuclear receptor gene into the pronucleus of a zygote derived from said animal of a), said zygote containing a homozygous disruption of the endogenous ERR $\alpha$  orphan nuclear receptor gene;
- 20 (c) transplanting said animal zygote into a pseudopregnant compatible animal;
- (d) allowing said zygote to develop to term;
- 25 (e) obtaining a founder animal carrying said transgene: and

(f) breeding said founder animal with a wild-type animal to obtain progeny that express said non endogenous ERR $\alpha$  orphan nuclear receptor transgene at levels sufficient to functionally complement the disrupted ERR $\alpha$  receptor activity.

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13. The method of claim 12, wherein said non-human transgenic animal is a mammal.

14. The method of claim 12, wherein said mammal is 10 a mouse, and wherein said non-endogenous ERR $\alpha$  transgene is a human ERR $\alpha$  gene.

15. A method for screening and identifying a compound 15 which modulates ERR $\alpha$  orphan nuclear receptor activity, the method including:

a) exposing the non-human transgenic animal of one of claims 5 to 7 to a candidate compound, and:

b) determining the activity of said ERR $\alpha$  orphan nuclear receptor in said animal,

20 wherein an increase in the receptor activity as compared to an unexposed non-human animal is indicative of a compound being capable of increasing ERR $\alpha$  orphan nuclear receptor activity, while a decrease in said receptor activity as compared to an unexposed non-human animal, is indicative of a compound being capable of decreasing ERR $\alpha$  orphan 25 nuclear receptor activity.

16. The method of claim 15, further comprising a determination of at least one parameter selected from the group consisting of: mass, body temperature, body fat content, fat to lean mass ratio, white adipose tissue deposits, basal metabolic rate, food intake, 5 hepatic synthetic functions, fasting serum triglyceride, serum glucose levels, level of expression of uncoupling protein mRNA in brown adipose tissue (BAT) and skeletal muscle, adipocyte volume in fat pads, lipogenesis, fatty acid esterification and fatty acid oxydation.

10 17. A method of identifying an agent which modulates fat and/or glucose metabolism *in vivo* comprising:

- administering an agent suspected of being a modulator of ERR $\alpha$  activity and/or level in an animal;
- measuring lipid and/or glucose levels in the animal 15 of step a) and comparing same with that of a control animal not having been administered said agent, wherein a difference in lipid and/or glucose levels of the animal of step a) as compared to that of the control animal, identifies said agent as a modulator of fat and/or glucose metabolism *in vivo*.

20 18. Method of identifying an agent which modulates fat and/or glucose metabolism *in vivo* comprising:

- providing a promoter operably linked to a selectable or assayable marker, said promoter being modulated by ERR $\alpha$ ;
- measuring or selecting for said marker in a 25 presence and in an absence of an agent suspected of modulating the

promoter modulating activity of  $ERR\alpha$ , thereby identifying an agent which modulates  $ERR\alpha$  activity wherein a difference in the transcriptional activity in the presence of said agent, as compared to that in the absence thereof, identifies said agent as a modulator of  $ERR\alpha$  activity:

- 5                   c) administering said agent identified in b) to a non-human transgenic animal according to one of claims 1 to 7; and
- d) measuring lipid and/or glucose levels in said animal of step c) and comparing same with that of a control animal, not having been administered said agent, wherein a difference in lipid and/or glucose levels of the animal of step c) as compared to that of said control animal identifies said agent as a modulator of fat and/or glucose metabolism *in vivo*.

15                   19. The method of claim 18, where the agent is obtained from a library of compounds.

20                   20. The method of claim 19, wherein the animal is a mammal.

20                   21. The method of claim 20, wherein said mammal is a mouse or human.

22. A modulator of fat and/or glucose metabolism *in vivo* identified by any one of the methods of claims 18, 19, 20 or 21.

23. A method of modulating fat tissue growth and/or weight gain, comprising:

5 a) administering to an animal an agent which modulates the promoter activity of a gene, wherein said promoter comprises cis-acting elements selected from the group consisting of:

- i) an estrogen response element;
- ii) TGA AGG TCA;
- iii) AGG TCA NNN TGA CCT; and
- iv) functional variants of i-iii)

10 such as to modulate the level of said gene, thereby modulating fat tissue growth and/or weight gain in said animal.

15 24. The method of claim 23, wherein said agent modulates said promoter activity of said gene, by decreasing a level and/or activity of ERR $\alpha$ .

20 25. The method of claim 24, wherein said agent is an antibody specific to ERR $\alpha$ , or an epitope-bearing portion thereof.

26. The method of claim 23, wherein said modulation of said promoter activity is effected by inhibition of ERR $\alpha$  synthesis.

25 27. The method of claim 26, wherein said agent comprises an antisense RNA, complementary to a nucleotide sequence encoding ERR $\alpha$ .

28. A method of determining whether an agent modulates fat tissue growth and/or weight gain in an animal comprising:

- a) providing a transcriptionally active preparation of ERR $\alpha$  or related factors and a DNA sequence comprising a promoter having a cis-acting sequence which modulates activity thereof by an interaction thereto of said ERR $\alpha$  and related factors;
- b) measuring said transcriptional activity of said promoter or of a binding of at least ERR $\alpha$  or related factors to said cis-acting sequence in a presence and in an absence of an agent suspected of modulating the transcriptional activity of said promoter or the binding of said factors to said cis-acting sequence, thereby identifying an agent which modulates transcription of said promoter and wherein a difference in the transcriptional activity and/or binding in the presence of said agent, as compared to that in the absence thereof identifies said agent as a modulator of transcription;
- c) administering said agent identified in b) to a non-human transgenic animal according to one of claims 1 to 7; and
- d) measuring fat tissue growth and/or weight gain in the animal of step c) and comparing same with that of a control animal, not having been administered said agent, wherein a difference in fat tissue growth and/or weight gain of the animal of step c) as compared to that of the control animal identifies said agent as a modulator of fat tissue growth and/or weight gain *in vivo*.

25 29. The method of claim 28, where the agent is obtained from a library of compounds.

30. The method of claim 29, wherein the animal is a mammal.

31. The method of claim 30, wherein said mammal is 5 a mouse or human.

32. A modulator of fat and/or glucose metabolism *in vivo* identified by any one of the methods of claims 28, 29, 30 or 31.

10 33. A method of treating and/or preventing obesity, comprising administering to an obese animal, or an animal susceptible of becoming obese, an agent which modulates the promoter activity of a promoter comprising a cis-acting element selected from the group consisting of:

15 i) an estrogen response element;  
ii) TGA AGG TCA;  
iii) AGG TCA NNN TGA CCT; and  
iv) functional variants of I-iii)

wherein cis-acting element is capable of binding to ERR $\alpha$ .

20 34. The method of claim 33, wherein said agent reduces the level and/or activity of ERR $\alpha$ .

25 35. A method of determining whether an agent modulates obesity in an animal comprising:

a) providing a transcriptionally active preparation of

ERR $\alpha$  or related factors and a DNA sequence comprising a promoter having a cis-acting sequence which modulates activity thereof by an interaction thereto of said ERR $\alpha$  and related factors;

- 5        b) measuring said transcriptional activity of said promoter or of a binding of at least ERR $\alpha$  or related factors to said cis-acting sequence in a presence and in an absence of an agent suspected of modulating the transcriptional activity of said promoter or the binding of said factors to said cis-acting sequence, thereby identifying an agent which modulates transcription of said promoter and wherein a difference in the transcriptional activity and/or binding in the presence of said agent, as compared to that in the absence thereof identifies said agent as a modulator of transcription;
- 10        c) administering said agent identified in b) to a non-human transgenic animal according to one of claims 1 to 7; and
- 15        d) assessing obesity in the animal of step c) and comparing same with that of a control animal, not having been administered said agent, wherein a difference in obesity of the animal of step c) as compared to that of the control animal identifies said agent as a modulator of obesity *in vivo*.

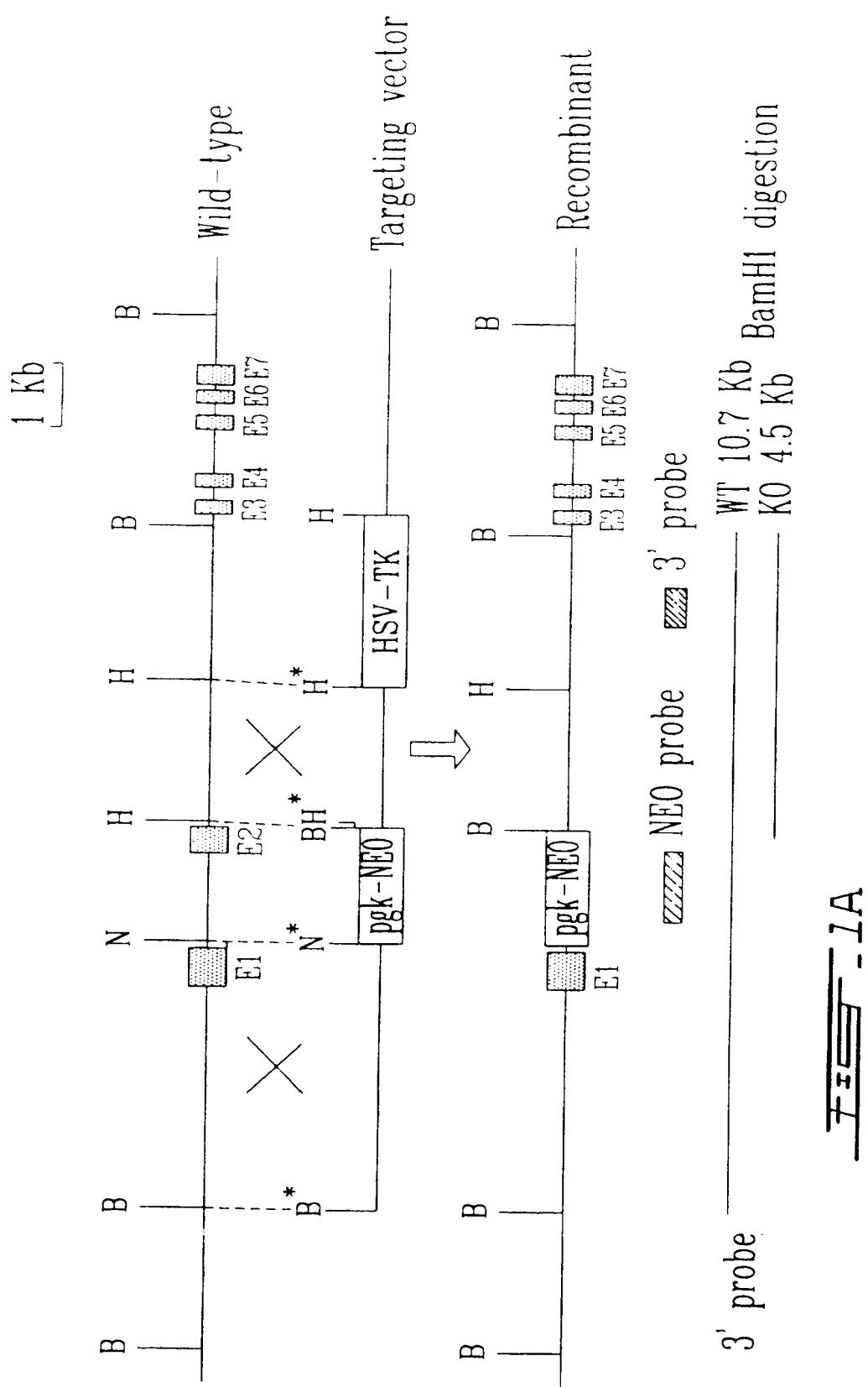
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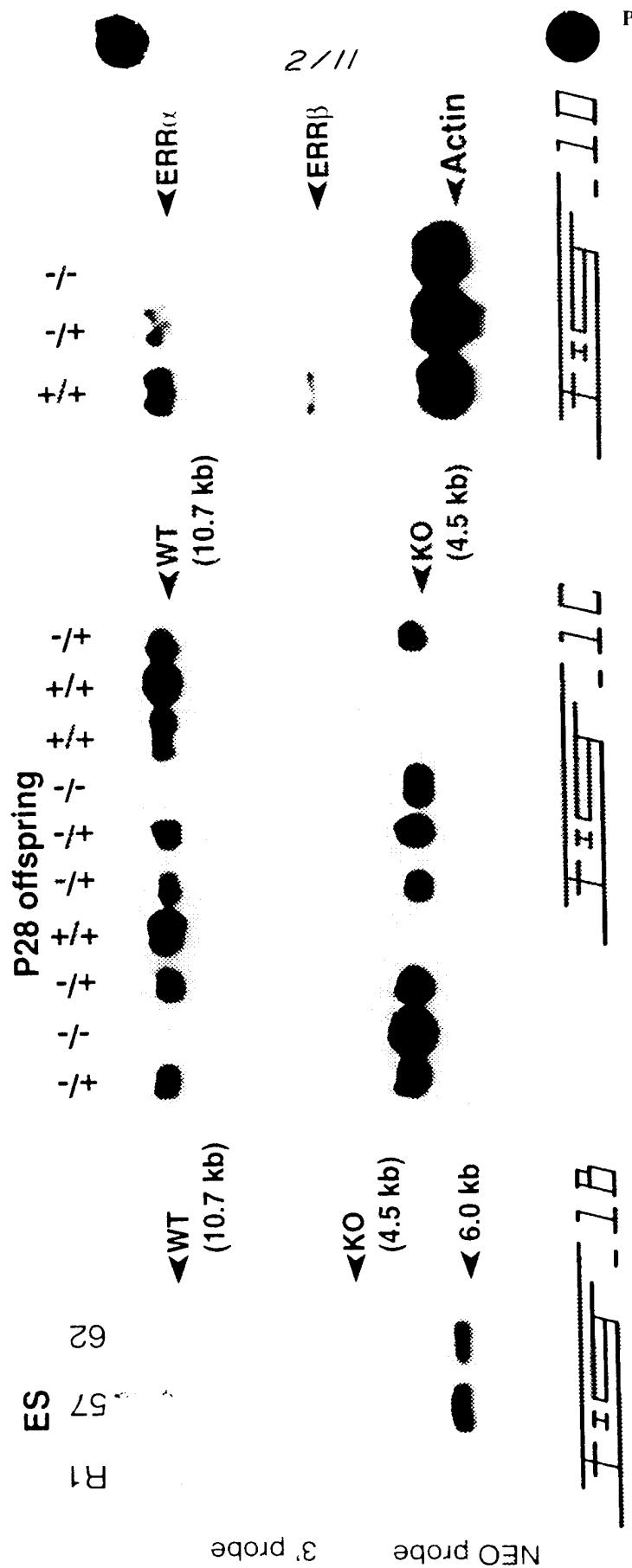
36. The method of claim 35, where the agent is obtained from a library of compounds.

25        37. The method of claim 36, wherein the animal is a mammal.

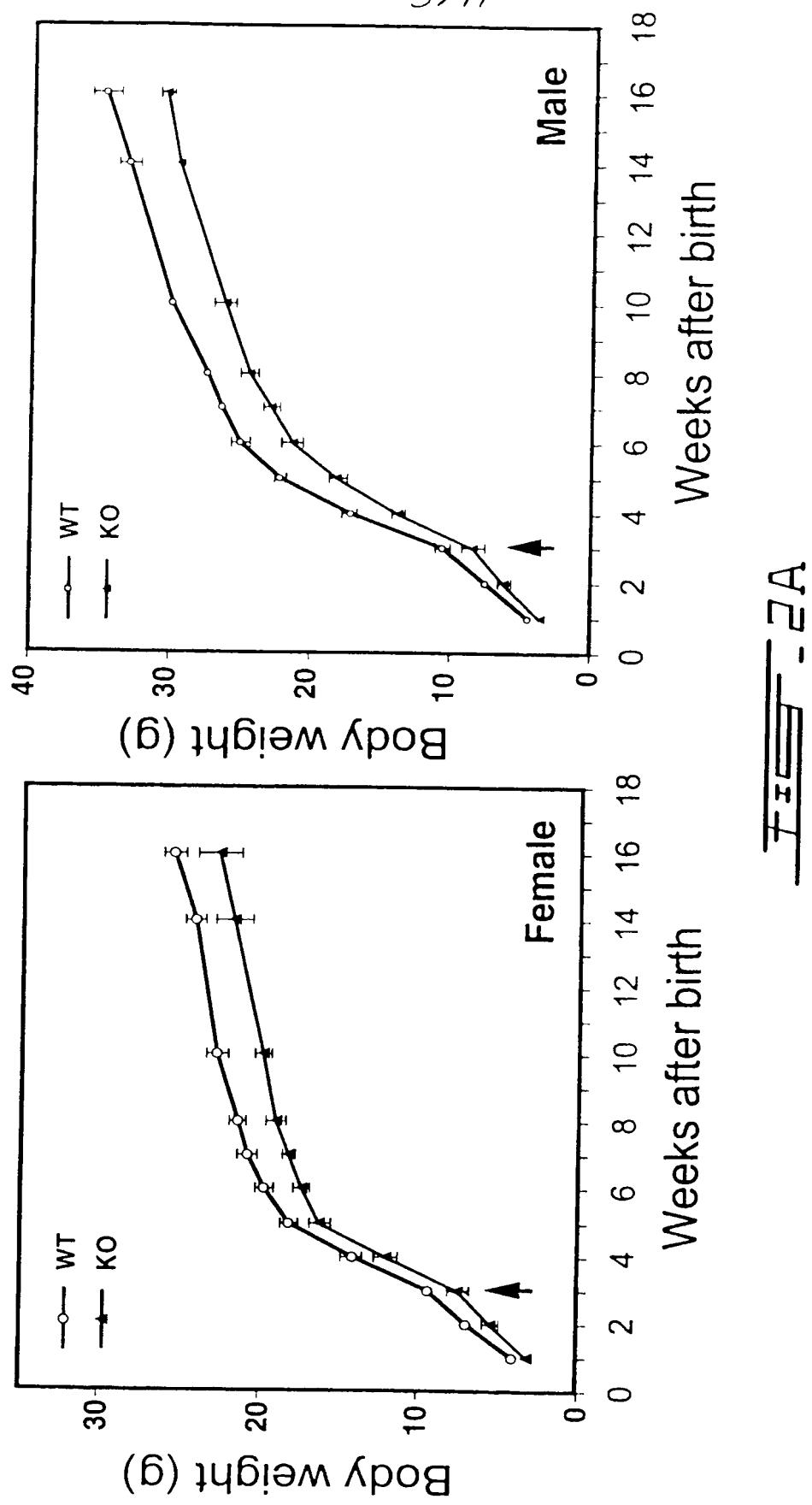
38. The method of claim 37, wherein said mammal is a mouse or human.

39. A modulator of glucose or fat metabolism *in vivo* 5 identified by any one of the methods of claims 35, 36, 37 or 38.

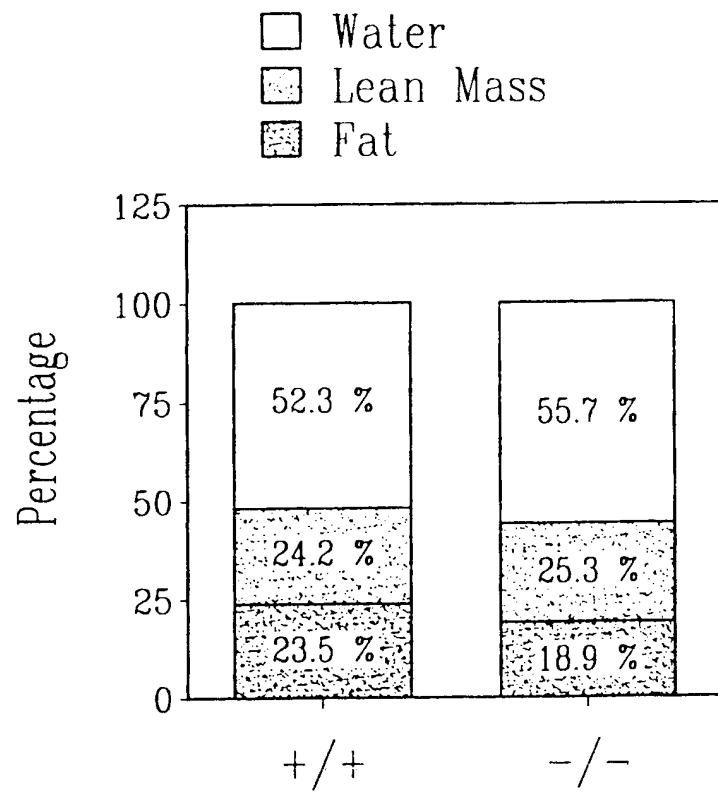




3/11



4/11

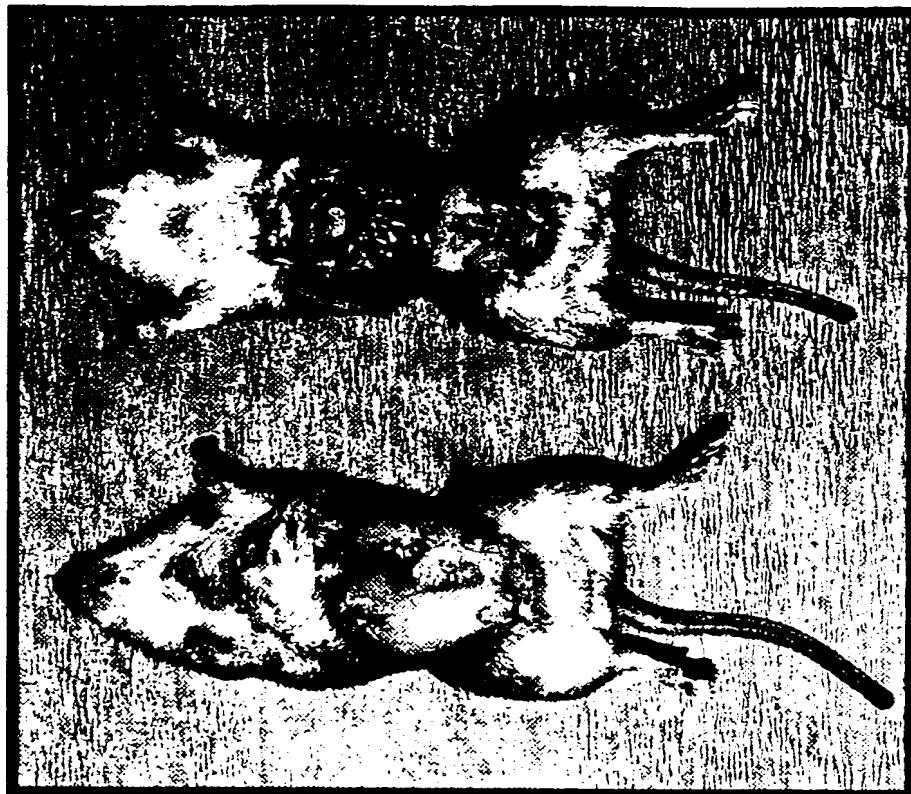


Fat / lean mass

0.30      0.23

 $p < 0.05$ 王士 - 28

5/11



-/-

+/\*

~~TELE~~ - 2C

-/-

+/\*

6/11

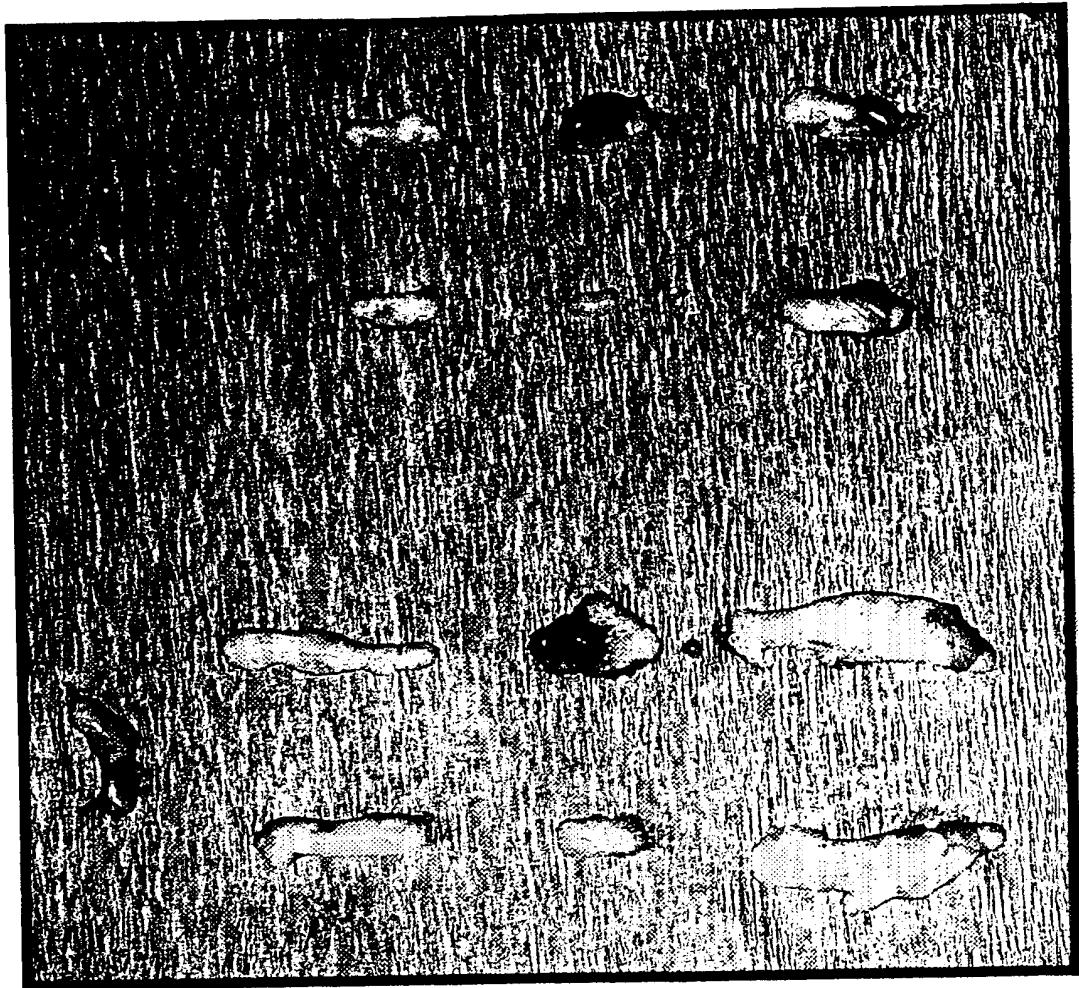


FIGURE - 20

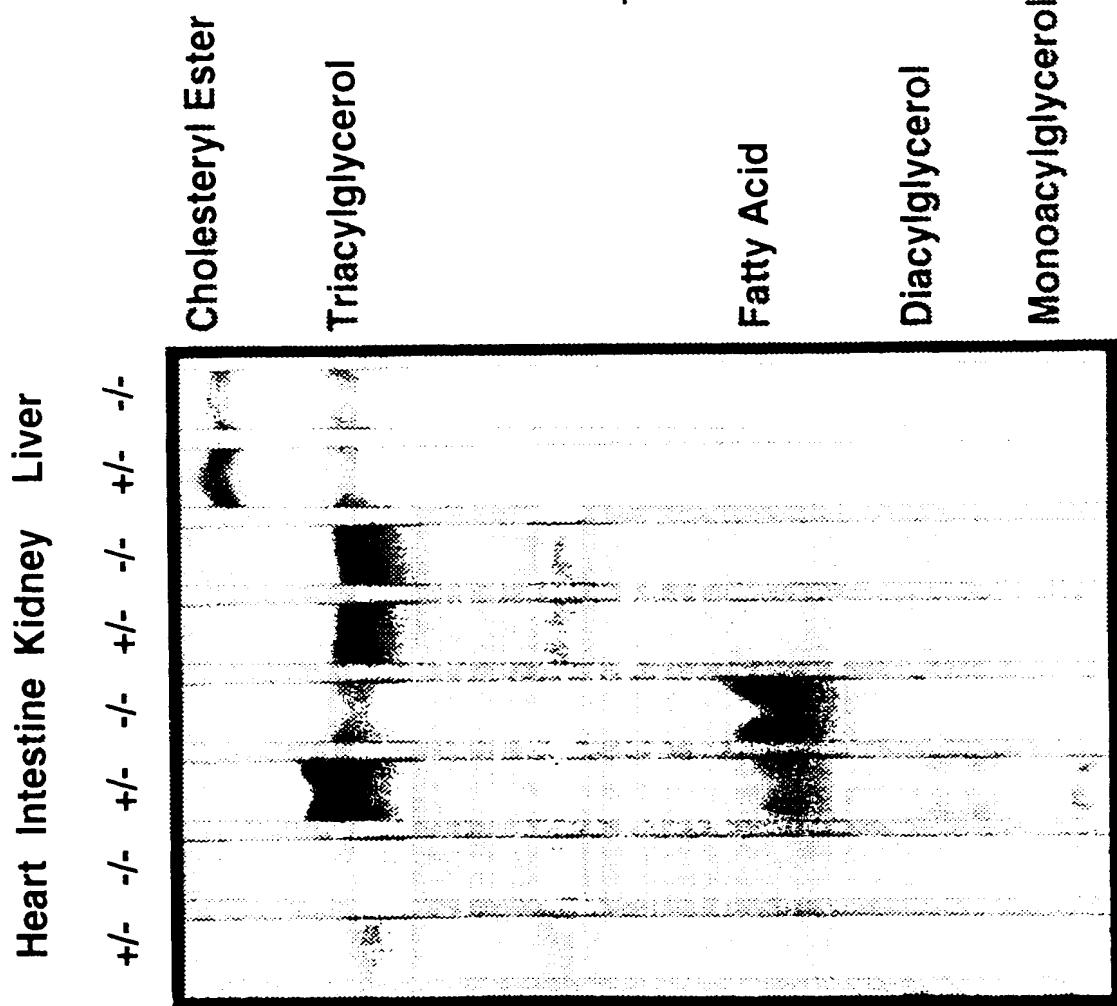
Dorsoscapular

Inguinal

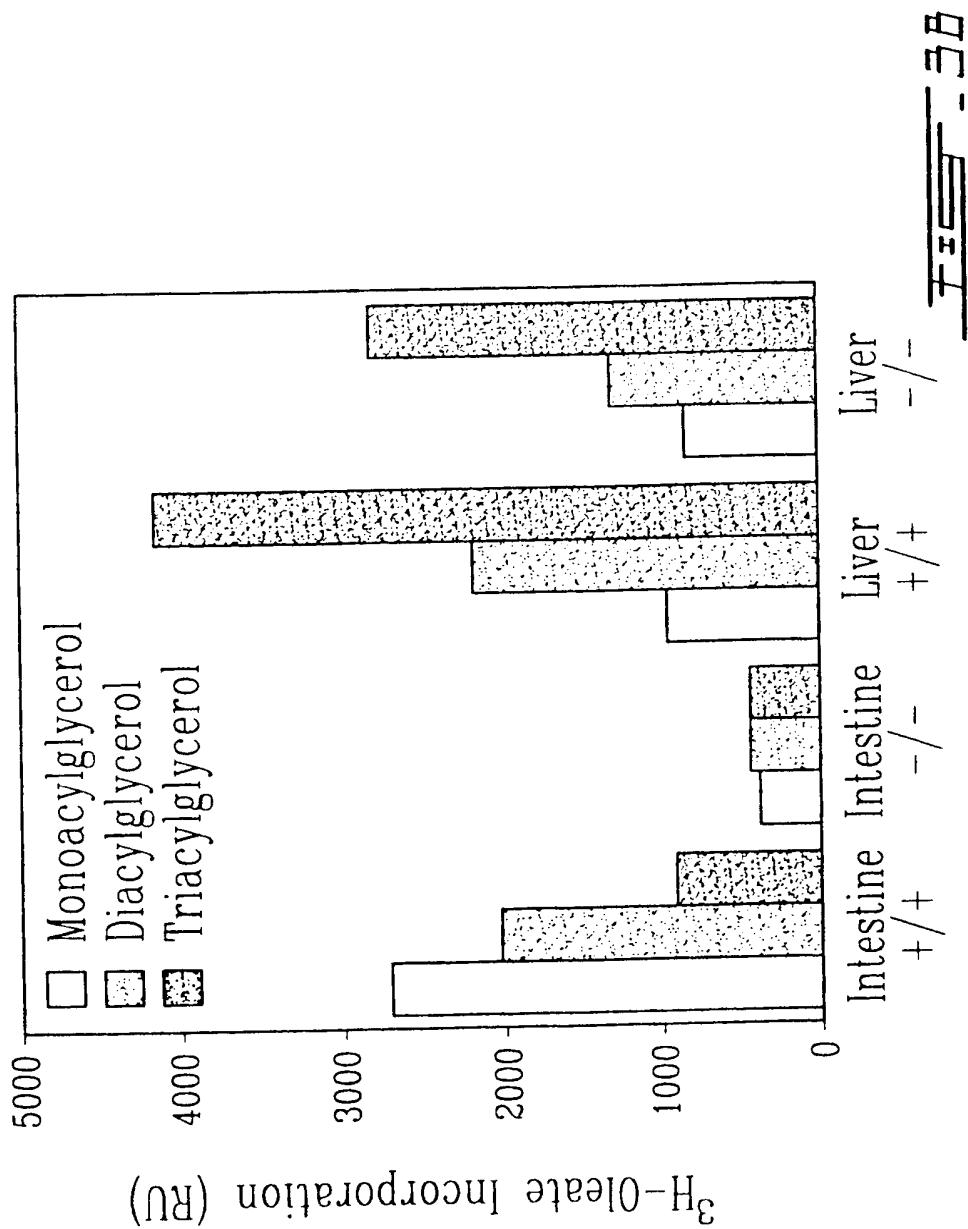
Perirenal

Epididymal

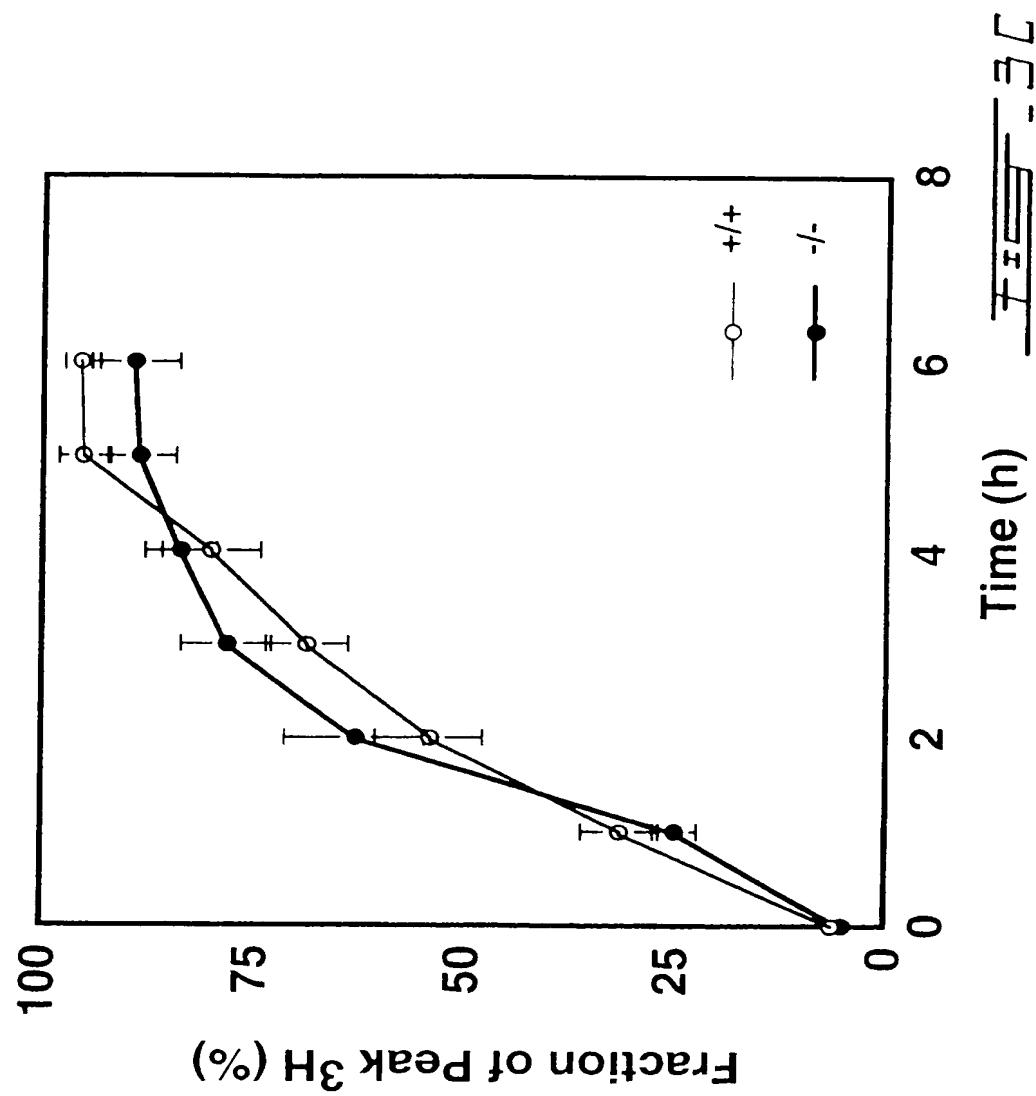
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9/16



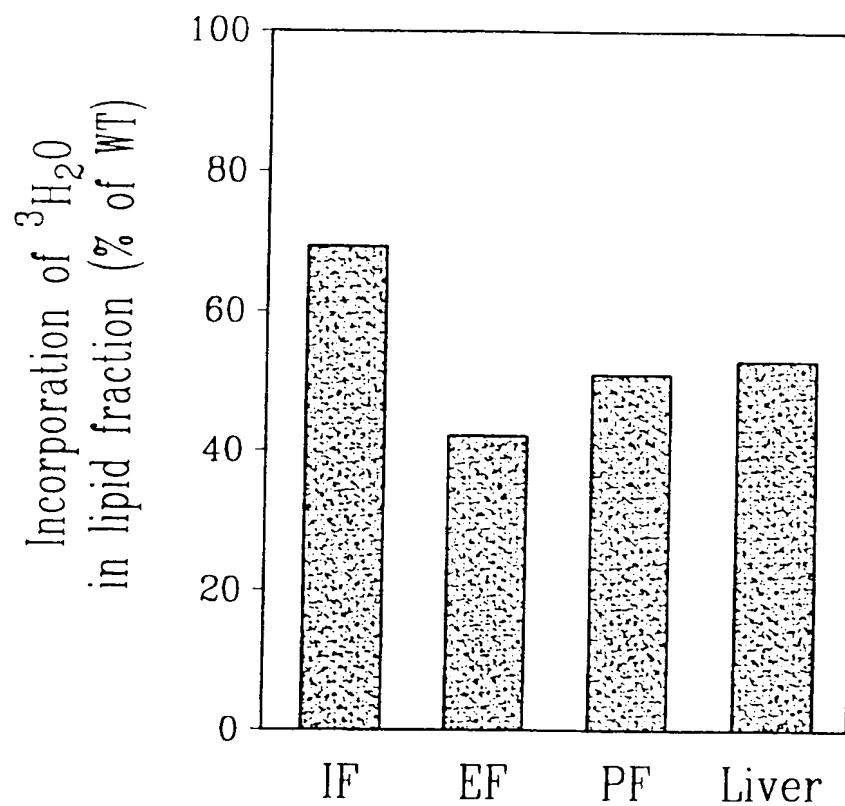
10/11

**ERR $\alpha$  WT**

**ERR $\alpha$  -**

~~FIG~~ - 4A

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Fig. 4B

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
17 August 2000 (17.08.2000)

PCT

(10) International Publication Number  
**WO 00/47735 A3**

(51) International Patent Classification<sup>1</sup>: **C12N 15/12**, 15/11, 5/10, A01K 67/027, C07K 14/705, A61K 48/00, 49/00, G01N 33/50, C12Q 1/68, A61K 39/395, A61P 3/06

(74) Agents: DUBUC, Jean, H. et al.; Goudreau Gage Dubuc, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).

(21) International Application Number: PCT/CA00/00145

(81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 8 February 2000 (08.02.2000)

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

(26) Publication Language: English

Published:

— With international search report.

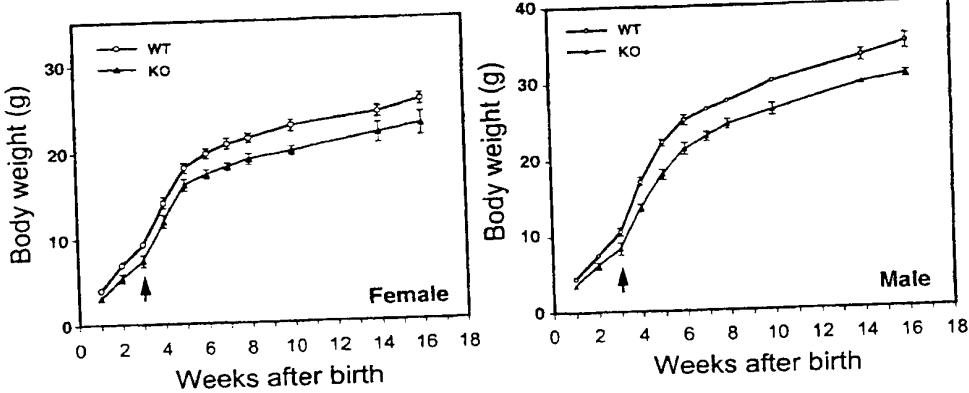
(30) Priority Data:  
60/119,024 8 February 1999 (08.02.1999) US

(88) Date of publication of the international search report:  
30 November 2000

[Continued on next page]

(54) Title: NON-HUMAN TRANSGENIC ANIMAL WHOSE GERM CELLS AND SOMATIC CELLS CONTAIN A KNOCK-OUT MUTATION IN DNA ENCODING ORPHAN NUCLEAR RECEPTOR ERR $\alpha$ lpha

**WO 00/47735 A3**



(57) Abstract: The present invention relates to a transgenic non-human mammal whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR $\alpha$ . More specifically, the invention relates to knockout mice whose germ cells and somatic cells contain a knockout mutation in DNA encoding orphan nuclear receptor ERR $\alpha$ . The invention further relates to such knockout non-human mammals which express an ERR $\alpha$  gene which is different from the endogenous gene which was disrupted. In a particular embodiment, the invention relates to a transgenic mouse having its endogenous ERR $\alpha$  gene disrupted and expressing human ERR $\alpha$ . As well, the invention relates to cell lines in which ERR $\alpha$  activity (and/or level) has been inactivated or augmented. The invention further relates to uses and methods of the transgenic animals of the present invention to select agents which modulate the expression and/or activity of ERR $\alpha$ , in particular in relation to fatty acid metabolism.

*For two-letter codes and other abbreviations, refer to the "Codes and Notes on Code and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/CA 00/00145

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7	C12N15/12	C12N15/11	C12N5/10	A01K67/027	C07K14/705
	A61K48/00	A61K49/00	G01N33/50	C12Q1/68	A61K39/395
	A61P3/06				

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N A01K C07K A61K G01N C12Q A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BRYANT H AND DERE W: "Selective estrogen receptor modulators: an alternative to hormone replacement therapy" PROC SOC EXP BIOL MED., vol. 217, no. 1, January 1998 (1998-01), pages 45-52, XP002142941 page 49, column 2, paragraph 2; figure 3; table 1 ---</p>	23,33
A	<p>SLADEK R ET AL: "The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene" MOL CELL BIOL., vol. 17, no. 9, September 1997 (1997-09), pages 5400-5409, XP002142942 cited in the application ---</p>	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

19 July 2000

02/08/2000

Name and mailing address of the ISA

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Authorized officer

Lonnoy, O

## INTERNATIONAL SEARCH REPORT

[REDACTED] onal Application No

PCT/CA 00/00145

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant claim No.
A	VEGA R ET AL: "A role for estrogen-related receptor alpha in the control of mitochondrial fatty acid beta-oxidation during brown adipocyte differentiation" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 50, 12 December 1997 (1997-12-12), pages 31693-31699, XP002142943 cited in the application ---	
A	EP 0 733 705 A (SALK INST FOR BIOLOGICAL STUDI) 25 September 1996 (1996-09-25) Experimental Section V ---	
A	LUO J ET AL: "Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta" NATURE., vol. 388, no. 6644, 21 August 1997 (1997-08-21), pages 778-782, XP002142944 ---	
P,A	WO 99 65486 A (PAN LYDIA CODETTA ;KE HUA ZHU (US); LI MEI (US); PFIZER PROD INC () 23 December 1999 (1999-12-23) -----	

INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 00 00145

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 22,32,39

Claims 22, 32 and 39 refer to a modulator of glucose and/or fat metabolism in vivo identified by any one of the described methods without giving a true technical characterization. Moreover, no such compound is defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such speculative claims the wording of which is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Original Application No

PCT/CA 00/00145

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0733705	A 25-09-1996	US 5071773 A AT 182173 T AU 616389 B AU 8238887 A DE 3752284 D DE 3752284 T EP 0287653 A JP 10004987 A JP 10108684 A JP 11137272 A JP 1500964 T WO 8803168 A US 5597705 A US 5534418 A US 5606021 A US 5312732 A US 5298429 A	10-12-1991 15-07-1999 31-10-1991 25-05-1988 19-08-1999 25-11-1999 26-10-1988 13-01-1998 28-04-1998 25-05-1999 06-04-1989 05-05-1988 28-01-1997 09-07-1996 25-02-1997 17-05-1994 29-03-1994
WO 9965486	A 23-12-1999	AU 4054799 A	05-01-2000